

PLASMA PROTEINS IN HEALTH AND DISEASE

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INTRODUCTORY REMARKS

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The topic plasma proteins encompasses a broad field of investigation, as is evident from the subject matter presented in this monograph. These proteins have been studied most intensively during the past 25 years; more intensively with the improved and refined methods of electrophoresis, immunology, and biochemistry. Although much work has been done on the various phases of plasma protein research there have been few opportunities for workers in this field to meet as a group and discuss problems and gain new knowledge and approaches to their specific problems. It was with this thought in mind that the conference on which this monograph is based was organized.

The complex and important field under review in this publication demands continued investigation from many approaches, and no doubt workers in one area enlighten and extend the comprehension of other investigators. The contributions to this monograph give a good indication of the current disciplines in this field and of the direction in which research is being intensified. It is anticipated that this monograph will bring forth a wide variety of problems and answer as many questions.

Part I. Dynamic and Biochemical Properties of Plasma Proteins

TWO PROTEINS OF HUMAN SERUM RELATED TO THE COMPLEMENT SYSTEM

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Complement is the heat-labile activity in serum that exerts a cytotoxic effect on antibody-coated cells. This is best demonstrated by the lysis of sensitized red cells. Complement consists of at least six components, all of which are serum globulins. They can be separated to a certain extent by ammonium sulphate fractionation¹ and by chromatography.² Such serum fractions, while containing the activity of only a single complement component, are physically and immunologically still highly complex. As a result, very little is known thus far about the chemical properties of individual components. Since they are serum proteins the use of methods that in recent years have greatly advanced serum-protein research may prove valuable also in their elucidation. Such methods include chromatography on ion exchange cellulose,³ preparative zone electrophoresis and zone ultracentrifugation,⁴ and immunoelectrophoresis.⁵ Undoubtedly isolation and characterization of individual components will be necessary before complement reactions or the role of complement in biology can be conclusively defined.

The investigations discussed in the present paper have their origin in studies on serum proteins. During the course of this work it became apparent that 2 of these proteins studied bore resemblance to complement components. One of these, referred to as 11S component,^{6,7} was found to be involved in the initial step; the other, designated β_{10} globulin,^{8,9} in the final step of immune hemolysis by human complement.

The 11S Component

The 11S component is a heat-labile protein of human serum that is able to precipitate soluble aggregates of γ globulin. Previous investigations have shown that rheumatoid factor, a pathological serum component with a strong affinity for γ globulin, forms precipitates with γ -globulin aggregates.^{10,11} When normal sera were tested these too were found to interact with aggregates. The extent of the precipitating action of normal serum was much smaller than that observed with pathological sera containing rheumatoid factor. However it was definite and, of more than 80 sera tested, all without exception gave this reaction. An important characteristic of the normal precipitating factor was its heat lability, and this precluded its identity with rheumatoid factor, which is a thermostable protein. Furthermore, by ultracentrifugal analysis, the sedimentation coefficient was found to be 11S, whereas rheumatoid factor is known to be a 19S protein.

The observed heat lability of the precipitating factor of normal serum suggested that it might be complement or one of the components of complement. This assumption was supported by previously reported observations according

to which complement is able to interact with soluble γ -globulin aggregates.^{12-14,9} Identity with the first component appeared most probable when the factor was found to be present in all reagents for the detection of complement components except that lacking the first. These findings were also noted and independently reported by Taranta.^{15,16} The observation however that the 11S component reacted with γ -globulin aggregates in the absence of bivalent cations rendered its identity with the first component unlikely.

For further investigation the precipitating factor was isolated from precipitates made with normal serum and γ -globulin aggregates in the presence of EDTA. More than 20 different preparations were obtained. Some of these consisted of more than 95 per cent of 11S (FIGURE 1); others contained in addition small amounts of 7S and 19S material. All preparations were active in the precipitation test with soluble γ -globulin aggregates (FIGURE 2). Electrophoretically the 11S component migrated as a γ globulin. However, quantitative immunological analyses using an anti- γ -globulin serum showed the 11S component to be distinct from γ globulin.

Because the 11S component reacted independently of bivalent cations, it was assumed that this protein was unrelated to complement. Using classical reagents for the detection of complement components, no activity could be detected in 11S preparations. It was surprising therefore to find strikingly reduced hemolytic activity in sera that had been depleted of 11S component. This was done by treatment with an excess of γ -globulin aggregates in the presence of EDTA, and subsequent removal of the aggregates by ultracentrifugation. After restoration of effective Ca^{++} and Mg^{++} levels such a serum may be designated an "R_{11S}." To investigate the loss of hemolytic activity, the titers of the 4 classical components were estimated by means of reagents. None was significantly reduced. When small amounts of 11S preparations were added to an R_{11S}, hemolytic activity was recovered almost completely. When the 11S preparation was heated at 56° C. for 45 min. prior to addition to an R_{11S}, this effect was not observed.

An attempt was made to prepare the intermediate complex "EA 11S," consisting of sensitized cells (EA) and 11S component (11S). Sensitized cells were treated with a preparation of 11S component for 5 min. at 37° C., then washed several times and, thereafter, exposed to a sublytic dose of an R_{11S} (EA 11S + R_{11S}). The ensuing hemolysis was analyzed kinetically and compared with lysis of sensitized cells by an R_{11S} + 11S (EA + R_{11S} + 11S). The results of this experiment illustrated in FIGURE 3 showed that the complex EA 11S does appear to exist as an intermediate product in immune hemolysis. An unexpected and thus far unexplained finding was a slight delay in the hemolysis of EA 11S cells as compared to that of EA cells.

Undoubtedly the 11S component fulfills a definite function in immune hemolysis by human complement. The evidence presented suggests that it participates in the initial step preceding the classical first component. The question remains to be answered: In what capacity is the 11S component involved? Does it represent a new component of complement, or an antibody or antibody not yet defined that is neither complement nor antibody?

Supposing that the 11S component were an antibody, its effect on hemolysis

could best be explained by assuming that it represents a natural antibody to sheep cells, and that the hemolysis experiments were performed with insufficiently sensitized cells. Only in a limited antibody system does further addition of antibody lead to increased hemolysis. The cells employed in all he-

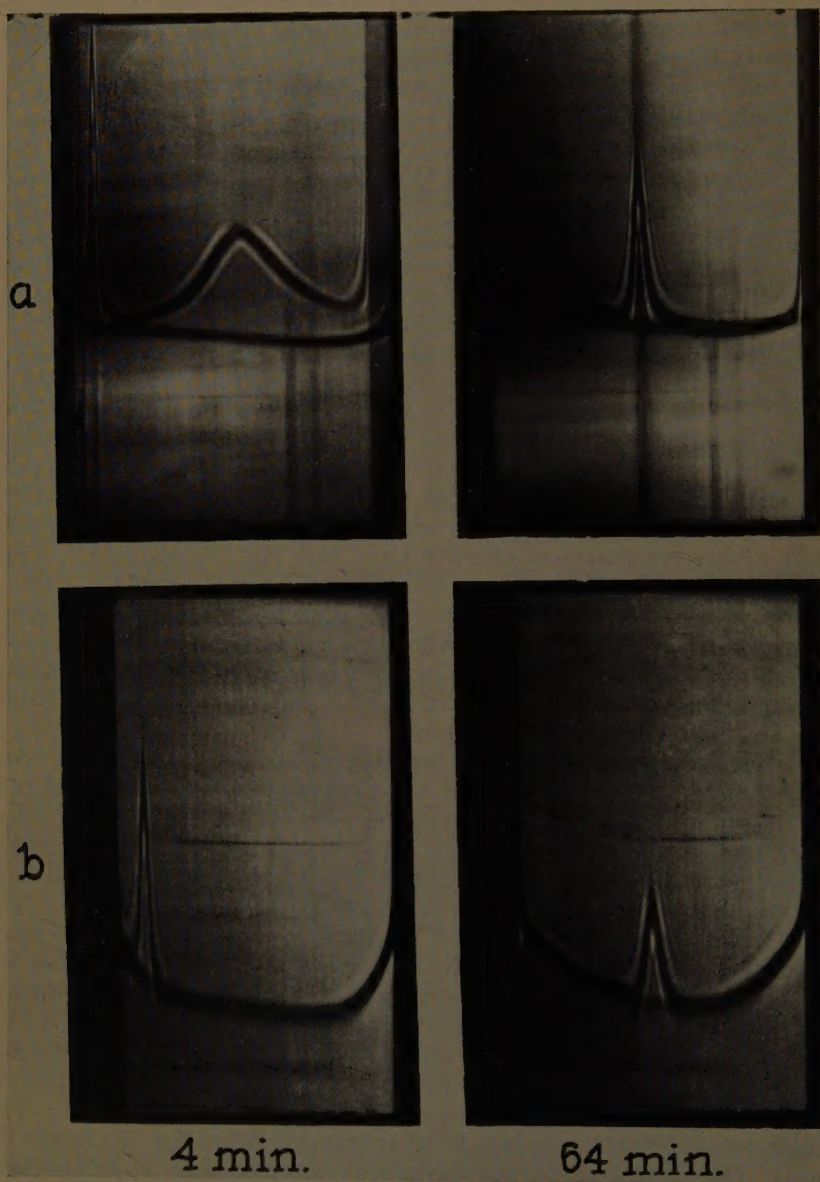


FIGURE 1. Ultracentrifugal patterns (a) of a dissolved precipitate formed by normal human serum upon addition of soluble γ -globulin aggregates in the presence of EDTA, and (b) of the isolated precipitating factor (11S component).⁷

molysis experiments, however, were optimally sensitized so that no limited antibody condition prevailed. Furthermore 11S preparations failed to sensitize sheep cells for lysis by guinea pig complement.¹⁷ Finally, it is improbable that a protein that is markedly heat-labile represents an antibody, especially if it differs in ultracentrifugal and immunological characteristics from known types of γ globulin.

Could the 11S component be a factor that aids the attachment of the first component to antibody molecules or that functions as a link between the two?

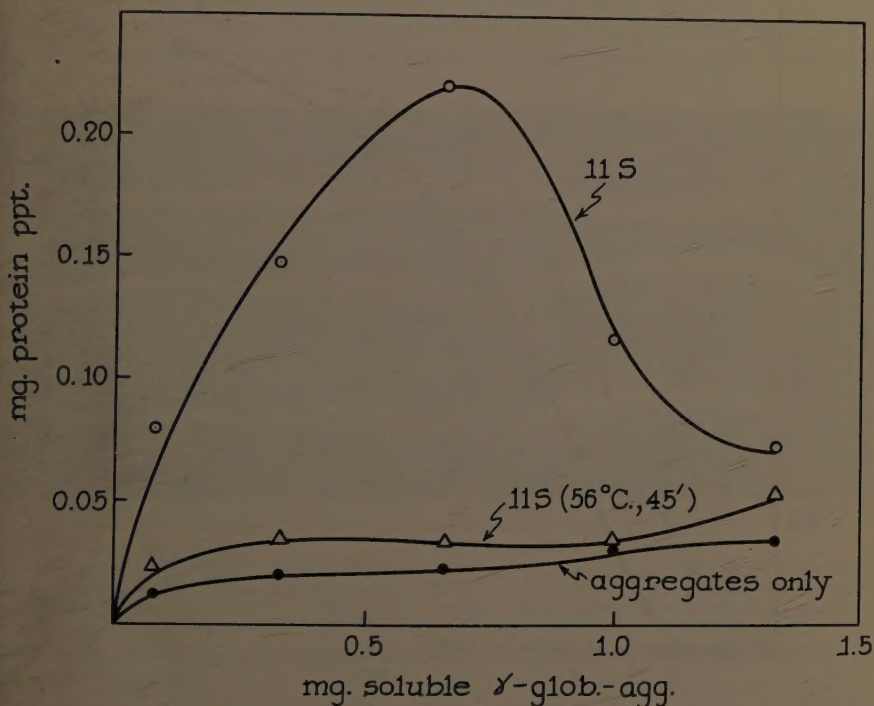


FIGURE 2. Precipitin curves of purified 11S component with soluble aggregates of γ globulin, showing also the effect of heating upon the precipitin reaction. Each tube contained 4 μ g. of 11S component.

Recently it has been shown that for the fixation of guinea pig complement to bovine antibody a heat-labile euglobulin is required that occurs in fresh bovine serum. This euglobulin is present in the reagents for the detection of complement components, except that lacking the first component.¹⁸ Similar observations have been made with avian sera.¹⁹ Whether or not this heat-labile factor is identical with the first component has not been investigated by the cited authors. The possibility exists that the factor present in bovine and avian sera constitutes an analogue to the 11S component of human serum. Further investigations are needed to decide whether this is the case and whether the 11S component represents a new component of human complement.

β_{1C} -Globulin

This serum protein was first recognized by immunoelectrophoresis. It is electrophoretically the slowest of the β_1 globulins. Being a relatively labile component, it tends to change to a protein with greater electrophoretic mobility and smaller molecular weight. The altered form of β_{1C} globulin* that is called β_{1A} globulin is a stable protein that does not undergo further spontaneous change. Formation of β_{1A} globulin occurs on aging of serum (FIGURE 4). At

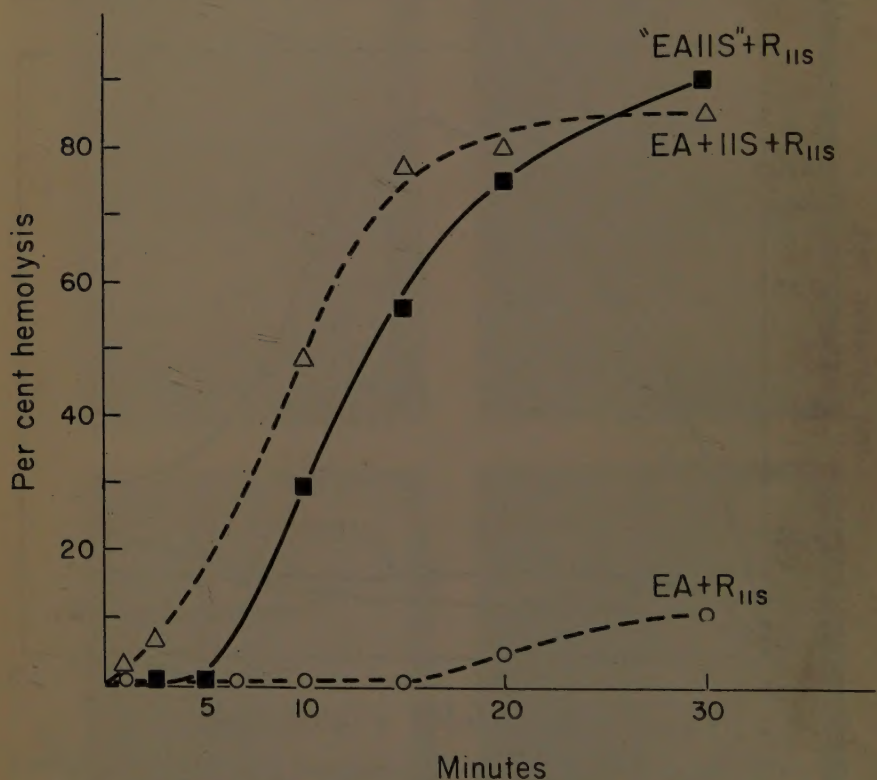


FIGURE 3. Kinetic analysis of hemolysis of "EA 11S" cells by an R_{11S}, and of EA cells by an R_{11S}, and by an R_{11S} plus 11S.

1° C. all of the β_{1C} globulin of a given serum is converted to β_{1A} within 4 weeks, and at 37° C. in 1 to 2 days. Conversion occurs within minutes on treatment of serum with antigen-antibody complexes at 37° C. Soluble aggregates of γ globulin, zymosan, endotoxin, and certain brands of dextran and of agar have the same effect. All of these materials have in common the property of inactivating hemolytic complement. It thus appeared that β_{1C} globulin represented one of the components of complement. Since the reaction was found to depend on a higher temperature and not to proceed at 0° C., β_{1C} globulin

* According to Heremans,²⁰ β_{1C} globulin is identical with an immunoelectrophoretic component called by Scheidegger²¹ " β_{2B} ."

could be related only to the third component, and not to the first, second, and fourth.

H. J. Rapp has furnished evidence that the third component (C_3') of guinea pig complement is composed of two² or even three²² factors. Leon²³ has shown C_3' of human serum to consist of three subcomponents. Previously Leon²⁴ had found C_3' of human serum to be inactivated by hydrazine, which indicated hydrazine sensitivity of at least one of the C_3' subcomponents. When β_{IC} globulin was treated with hydrazine it was found to change rapidly to β_{IA} globulin.¹⁷ As will be seen below, β_{IC} globulin does constitute a hydrazine-sensitive factor of the third component of complement.

The β_{IC} and β_{IA} globulins were isolated from fresh and aged serum respectively by chromatography on TEAE cellulose. The final products gave single pre-



FIGURE 4. Immunoelectrophoretic presentation of β_{IC} and β_{IA} globulin in different samples of whole serum. (a) Fresh serum; (b) aged serum (stored at 1° C. for four weeks); and (c) serum stored at 1° C. for 10 days. The arrows indicate the arcs of β_{IC} and β_{IA} globulin respectively. Patterns similar to (b) were obtained after incubation of fresh serum at 37° C. with either immune precipitates, soluble γ -globulin aggregates, zymosan, or hydrazine.

cipitin lines when tested by Ouchterlony's diffusion-in-gel method with an antiserum to whole human serum. In this test β_{IA} globulin proved antigenically slightly deficient as compared to β_{IC} globulin. Carbohydrate analyses showed that both components are glycoproteins containing approximately 2.7 per cent total carbohydrate in the form of neutral hexoses, hexosamine, and neuraminic acid. Ultracentrifugal examination revealed significantly different s-rates that were found to be 9.5S for β_{IC} globulin and 7S for β_{IA} globulin (FIGURE 5). Evidently the 2 proteins differ in molecular weight.

To study the activity of β_{IC} globulin, highly purified preparations were tested for their effect on the substrate of the third component. This is a sensitized cell that has interacted with the first (C_1'), fourth (C_4'), and second (C_2') component of complement ($EAC'_{1,4,2}$). At 37° C., these cells rapidly revert to the $EAC'_{1,4}$ state and thus lose their ability to react with C_3' .²⁵ In the presence of β_{IC} globulin, $EAC'_{1,4,2}$ cells were found not to decay, but to remain fully

reactive. It is probable that $EAC'_{1,4,2}$ cells and β_{1C} globulin combine to form a stable intermediate complex. As has been pointed out by Mayer,²⁵ the extent of hemolysis depends on the outcome of a race between the forward reaction, $EAC'_{1,4,2} \rightarrow E^*$ and the backward reaction, $EAC'_{1,4,2} \rightarrow EAC'_{1,4}$. Since β_{1C} globulin prevents the latter reaction it should have a promoting effect on hemolysis. In fact in a limited complement system a linear relation was found between the amount of β_{1C} globulin added and the degree of hemolysis ensuing (FIGURE 6). The activity of β_{1C} globulin was destroyed by treatment

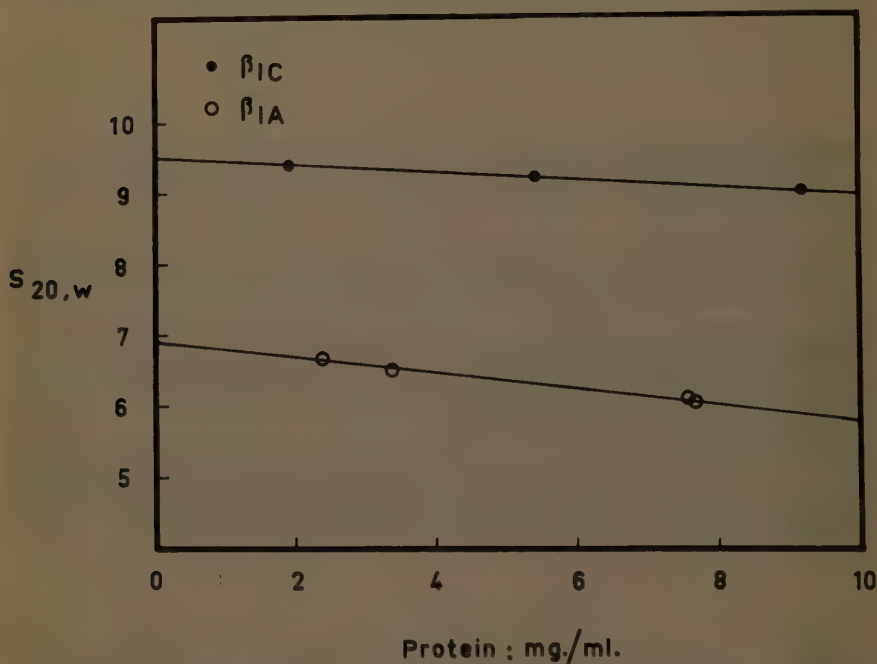


FIGURE 5. Sedimentation coefficient of β_{1C} and β_{1A} globulin at various protein concentrations. By least square analysis, $y = -0.05x + 9.5$ for β_{1C} , and $y = -0.11x + 6.9$ for β_{1A} .⁸

with hydrazine. At 37° C., 0.05 *M* hydrazine effected the destruction of 90 per cent activity within 20 min.¹⁷

It has thus far been assumed without question that the described hemolytic activity resides in β_{1C} globulin. The possibility exists however that the activity is not associated with β_{1C} globulin, but with an undetected contaminant present in these preparations. Although this possibility cannot be excluded completely, there is evidence against it. For instance, an excellent correlation was observed between inactivation of β_{1C} globulin and conversion to β_{1A} globulin. This was found both during aging of β_{1C} globulin and upon inactivation with hydrazine.

The finding that β_{1C} globulin was changed to β_{1A} globulin during incubation of serum with antigen-antibody complexes suggested that it was inactivated

in the process of this reaction but not fixed. Experiments with antisera prepared against fixed human complement showed, however, that part of this protein does combine with immune aggregates. Such antisera against fixed human complement have been prepared by Ellis and Gell,²⁶ Peetom *et al.*,²⁷ and in this laboratory. They contain antibodies against a number of serum proteins, the main portion of antibody being directed against a β globulin that by means of a specific antiserum could be identified as β_{1C} globulin (FIGURE 7).

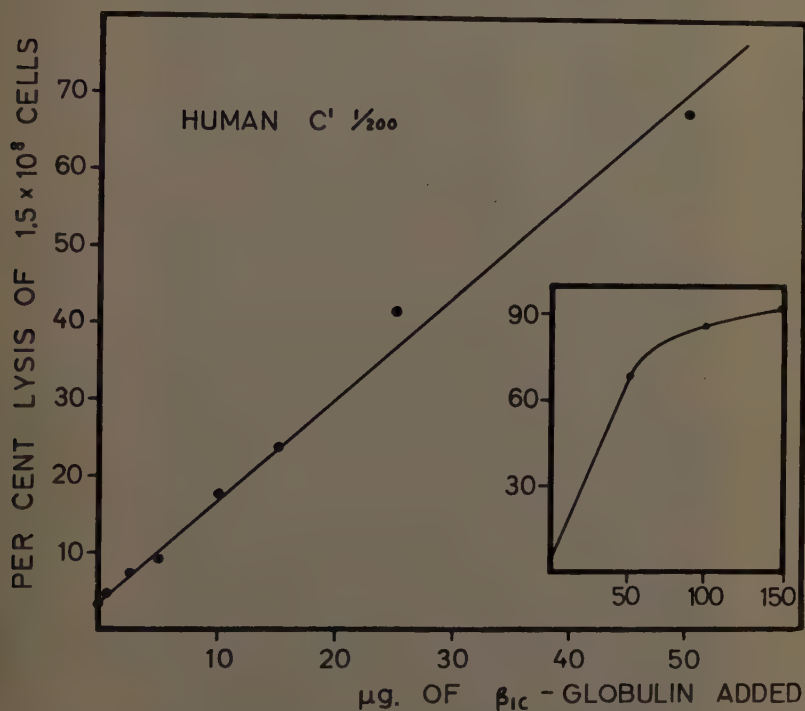


FIGURE 6. Quantitative relation between the final degree of hemolysis in a limited complement system and the amount of β_{1C} globulin added to this system.⁹

Summary

Two proteins, participating in immune hemolysis by human complement, have been isolated and partially characterized.

One protein was isolated from precipitates obtained by addition of soluble γ -globulin aggregates to serum in the presence of EDTA. The sedimentation coefficient of this protein was found to be 11S. The electrophoretic mobility was similar to that of γ globulin. Its activity proved to be heat labile. Evidence is presented that the 11S component is distinct from any of the classical components of complement, and that it is involved in the initial stage of complement action.

The other protein, β_{1C} globulin, is a glycoprotein with a sedimentation coefficient of 9.5S. Upon interaction of serum with immune aggregates at 37° C.,



FIGURE 7. Demonstration of antibodies to β_{1c} globulin in an antiserum to complement. Immunoelectrophoretic patterns of human serum developed with (a) antiserum to whole human serum; (b) antiserum to β_{1c} globulin; and (c) antiserum to complement.

this protein is changed to a component, β_{1A} globulin, with greater electrophoretic mobility and lower s-rate. Part of the protein is taken up by antigen-antibody complexes, while another portion remains in the supernatant. β_{1C} globulin was shown to be the hydrazine sensitive part of the third component of complement.

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THE BIOSYNTHESIS OF PLASMA PROTEINS*

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The biosynthesis of plasma proteins in mammalian organisms differs in several respects from the biosynthesis of other proteins. These are: (1) extracellular occurrence, which requires that plasma proteins diffuse through the cell walls; (2) synthesis and degradation at separate sites; and (3) a time lag. The plasma protein of other species have not been thoroughly studied. Such similarities as the requirements for ribonucleic acid, both soluble and insoluble, activating enzymes and the relation of amino acid sequence to DNA structure will not be discussed here. The synthesis of all major components with the exception of the gamma globulins takes place in the liver.

Due to their extracellular occurrence, their quantity, and their accessibility *in vivo*, plasma proteins have been the objects par excellence of study of protein metabolism, and the degradation and synthesis of plasma proteins represent the most clear-cut example of protein turnover. The kinetics of this process have been thoroughly studied, and the primary process, namely the degradation of plasma proteins, seems to be of the first order.¹ The synthesis appears to be regulated to compensate for the degradation by a mechanism at present unknown. Other processes such as slow equilibration of intra- and extravascular proteins complicate the kinetic analysis (FIGURES 1 and 2) and, since by necessity such experiments are always carried out with isotopic tracers, the storage of labeled amino acids in the form of body proteins makes the analysis even more difficult. Thus after feeding of several labeled amino acids together the rates with which the isotope concentrations change with time is different for individual amino acids (FIGURES 3 and 4). On the other hand, either after transfusion of labeled plasma or after continuous feeding of labeled amino acids, storage is negligible and the rates for different amino acids are identical (FIGURES 5 and 6). The rates are similar for different isotopic tracers (FIGURE 7).

It is at present not clear whether this kind of protein turnover applies in the same fashion to intracellular proteins. The synthesis of intracellular proteins can be shown to occur by means of isotopic tracers, but the rate of synthesis seems to parallel at least qualitatively the rate of cell division. Protein synthesis is extensive in such organs as liver and intestine, which show a high cell division rate and are extremely low in muscle and brain where cell division is absent. A recent analysis of this matter in ascites tumor cells *in vivo* with heavy oxygen (O^{18}) as a tracer (TABLE 1), showed that the rate of amino acid uptake is larger than could be accounted for by the increase in cell mass and that, therefore, protein synthesis over and above that due to cell division had occurred, that is, that in these cells proteins turn over in addition to growth.² Ascites tumor cells lend themselves quite well to this kind of investigation since they divide sufficiently rapidly to make reasonable accuracy

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in such experiments possible. In microorganisms turnover also occurs in addition to growth. However in this case the rate of turnover decreases if the division rate increases.³

If one assumes that proteins "wear out"—that by slight alterations of the molecular structure, proteins might become nonfunctional—they can then be

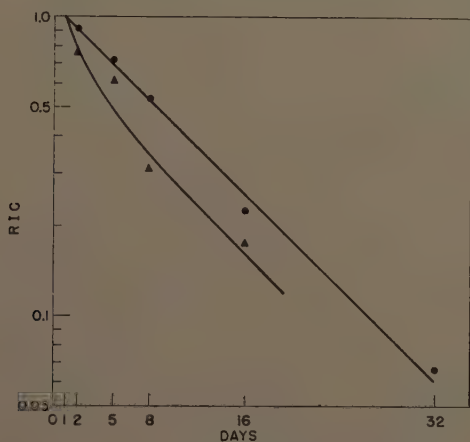


FIGURE 1. Change of 1-C¹⁴-glycine (●), injected 5 days before zero time, and 1-C¹³ glycine (▲), injected at zero time, concentrations of plasma albumin with time. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

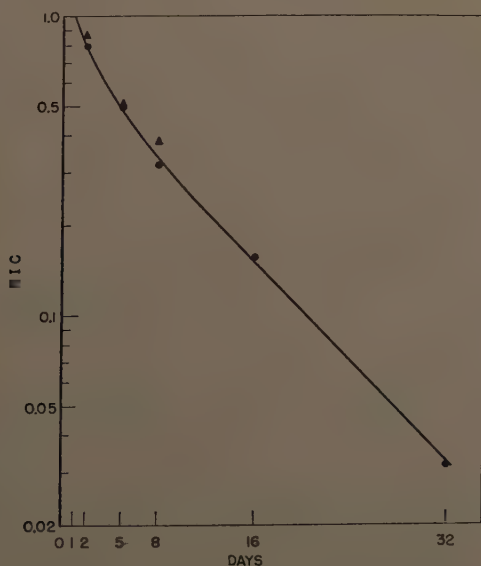


FIGURE 2. Change of isotope concentrations of plasma albumin after transfusion of 1-C¹⁴-glycine, 1-C¹³-glycine doubly labeled plasma at zero time. Labeled plasma removed from experiment shown in FIGURE 1 on day one. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

assumed to be either replaced by turnover or diluted by growth. However it is not at all clear at this time why the rates of protein synthesis in different cells vary. It has been suggested that the plasma proteins may serve directly or indirectly as sources of amino acids in various organs. Thus the transport function of these proteins may be expressed as turnover in tracer experiments.

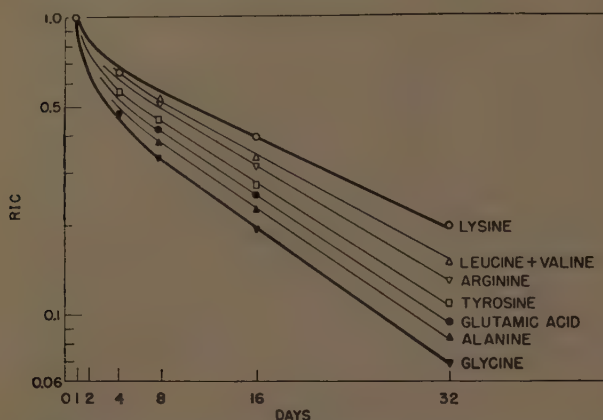


FIGURE 3. Change of isotope concentrations of various amino acids of plasma albumin with time. Labeled amino acids injected at zero time. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

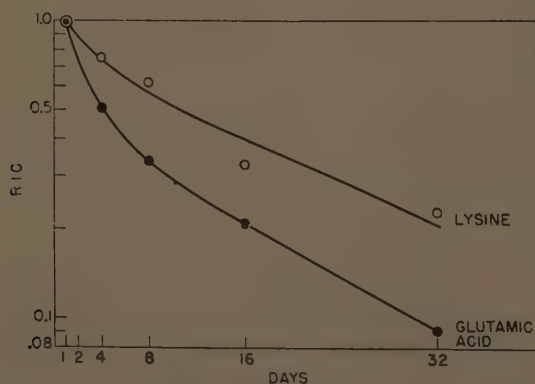


FIGURE 4. Isotope concentrations of plasma albumin after injection of labeled amino acids at zero time. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

One of the most remarkable aspects of plasma protein biosynthesis both *in vivo* and *in vitro* is the time lag⁴ that is observed to occur between the administration of labeled amino acids and their appearance in the plasma proteins (FIGURE 8). No such lag is ever observed for the incorporation of labeled amino acids into intracellular proteins. The lag is about 20 min. for small mammals at 37° C. and varies with the temperature with a Q_{10} of about 3 (FIGURE 9), as does the rate of incorporation (FIGURE 10). It has similar

values in cold-blooded animals. In some very elegant *in vitro* experiments, Peters showed that the microsomes were responsible for the lag.⁵ He could show that in liver preparations labeled amino acids are incorporated immediately upon addition into microsomal protein. However, the release of the serum albumin from the microsomes into the surrounding medium began only

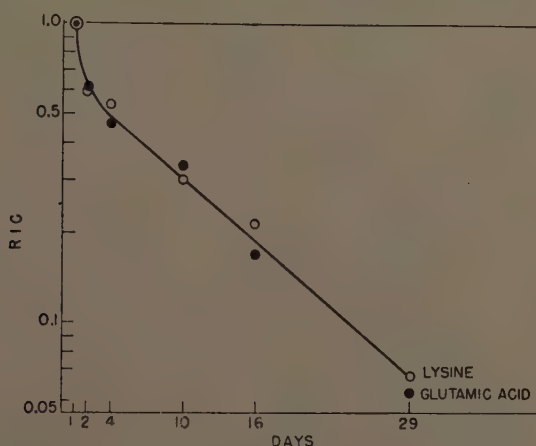


FIGURE 5. Change of isotope concentrations of plasma albumin after transfusion of lysine, glutamic acid doubly labeled plasma at zero time. Labeled plasma removed from experiment shown in FIGURE 4 on day one. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

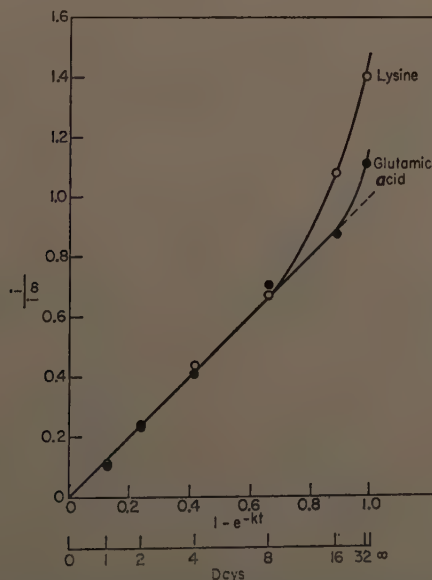


FIGURE 6. Change of isotope concentration of plasma albumin after daily administration of labeled lysine and glutamic acid. Reproduced by permission of *Biochim. et Biophys. Acta*.¹

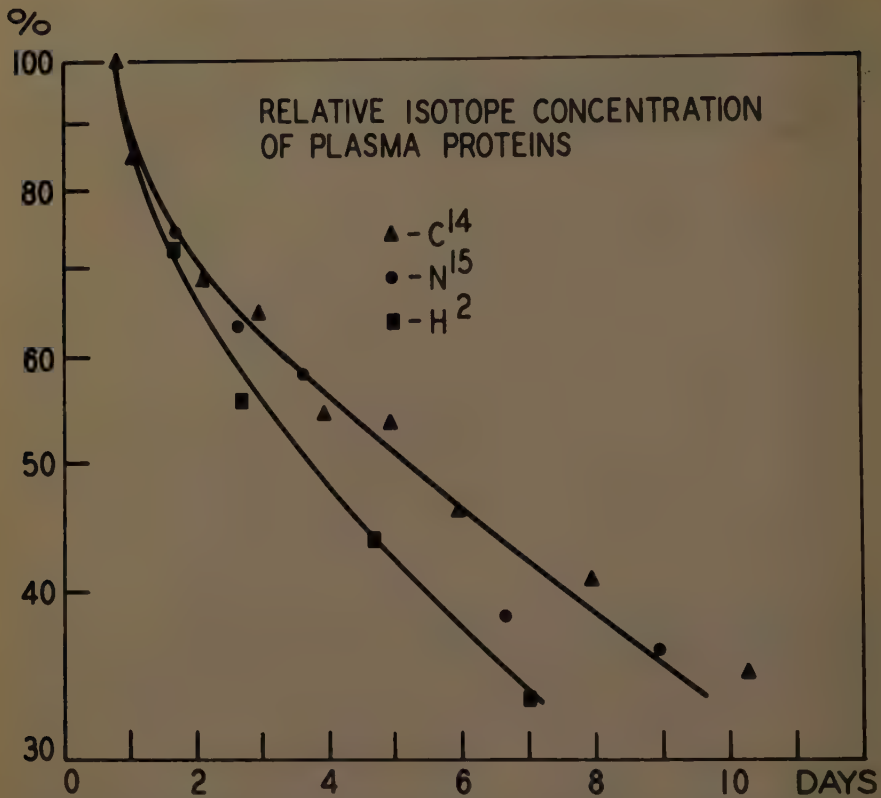


FIGURE 7. Change of isotope concentrations of plasma proteins after feeding a mixture of algae proteins labeled with C¹⁴ (▲), N¹⁵ (●) or H² (■). Reproduced by permission of *Biochim. et Biophys. Acta*.⁷

TABLE 1
INCORPORATION OF O¹⁸ INTO ASCITES TUMOR CELL PROTEIN FROM BODY WATER

Time (hours)	O ¹⁸ * (RIC)	Cell mass† (increase)	Turnover‡ (incorporation)
0.5	0.012	0.004	0.003
1	0.024	0.008	0.006
2	0.023	0.016	0.013
4	0.036	0.033	0.025
8	0.13	0.067	0.050
16	0.23	0.14	0.10
26	0.36	0.23	0.15

* In peptide bond.
† Calculated from mitotic index and generation time.
‡ Calculated turnover of liver protein.

about 20 min. later (FIGURE 11). The diffusion of plasma proteins through the cell walls at the site of synthesis does not seem to be associated with a time lag. A similar time lag was observed for the synthesis of the secretory proteins of the pancreas.⁶ The mechanism responsible for the time lag is unknown.

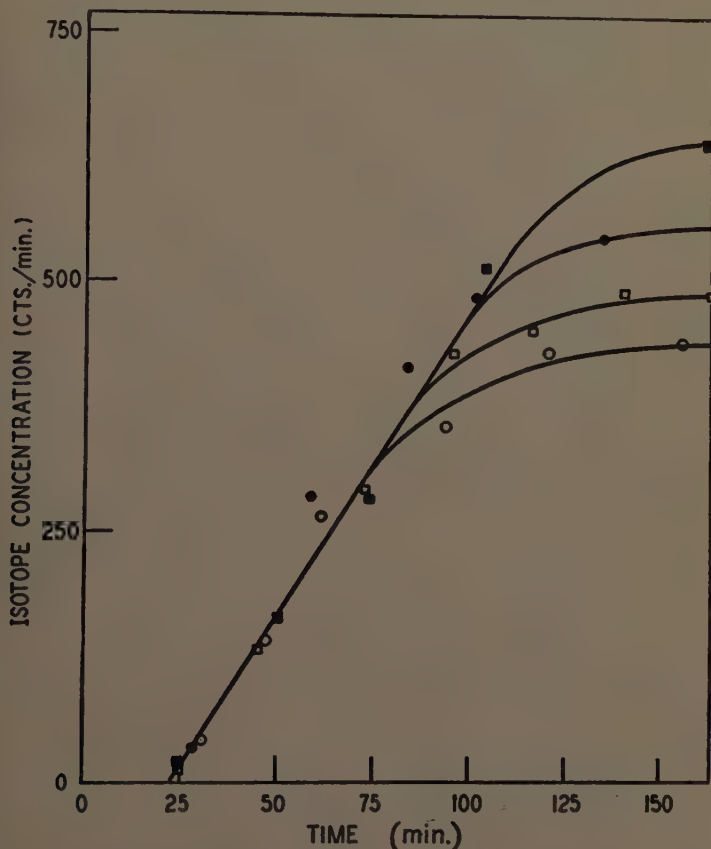


FIGURE 8. Incorporation of labeled amino acids injected at zero time in plasma proteins *in vivo*. Reproduced by permission of the *Journal of General Physiology*.⁴

An even more pronounced lag period is encountered in antibody synthesis. In rabbits with soluble antigens, the lag period is of a minimum of 60 hours' duration. While it is clear that this lag period is due to the reorientation of the cellular organization towards antibody production, its length is nevertheless unexplained. In the phenomenally related case of adaptive enzyme formation, no lag period of this magnitude is observed. Tracer experiments have shown that synthesis and storage of antibody protein during the lag period is quantitatively only of minor importance.⁷ Due to the wide but thinly spread distribution of the reticuloendothelial system throughout the mammalian

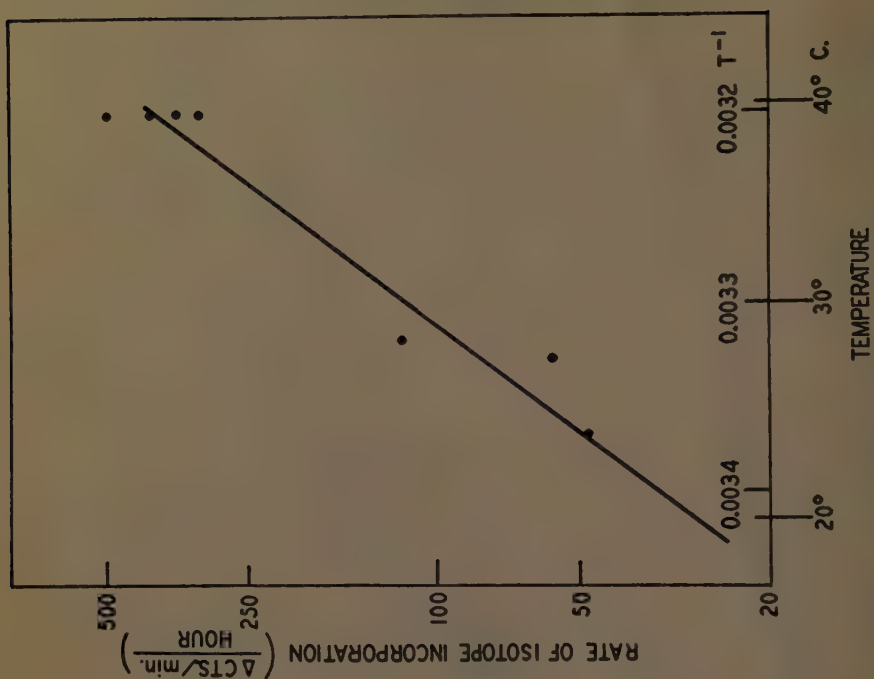


FIGURE 10. Temperature dependence of the rate of incorporation of labeled glycine into plasma proteins. Reproduced by permission of the *Journal of General Physiology*.

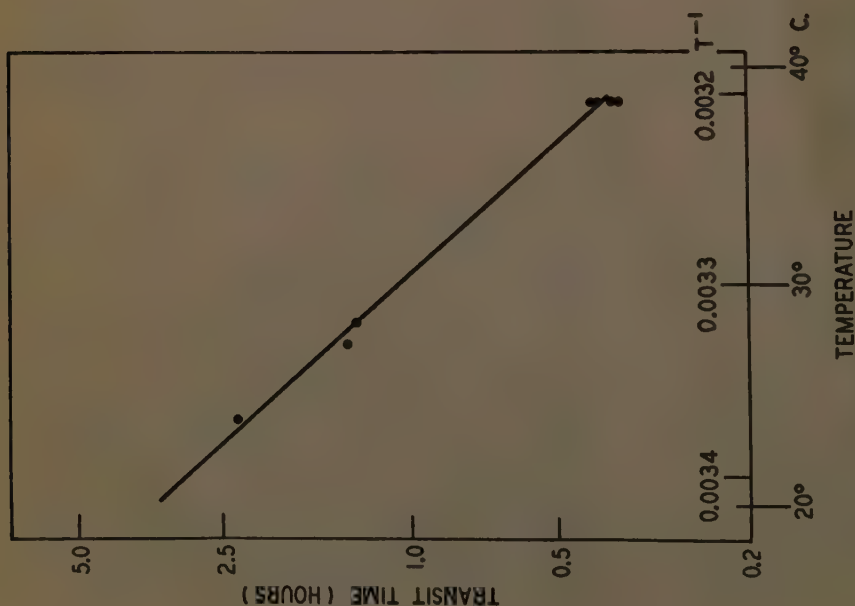


FIGURE 9. Temperature dependence of the time lag of incorporation of labeled glycine into plasma proteins. Reproduced by permission of the *Journal of General Physiology*.⁴

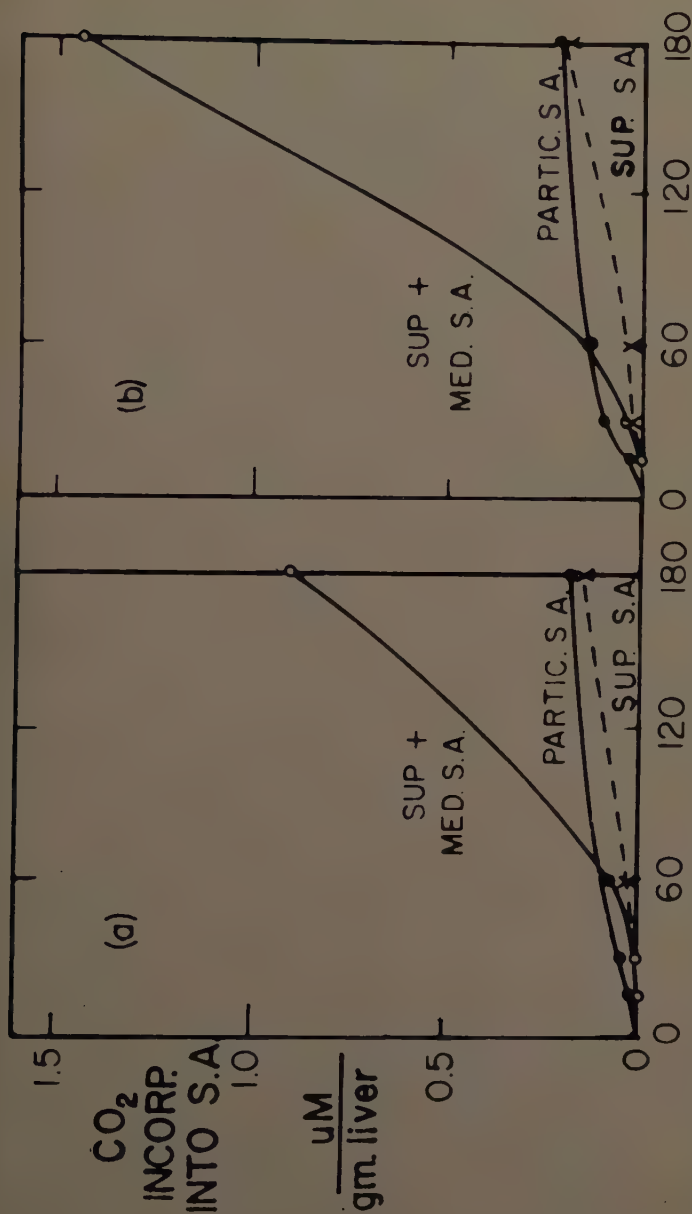


FIGURE 11. Time lag for incorporation of label into albumin of supernatant medium in liver slices *in vitro*. No time lag is observed for particle-bound albumin. Reproduced by permission of the *Journal of Biological Chemistry*.³

organism the isolation of unifunctional cellmasses for the *in vitro* study of antibody synthesis is difficult. It has therefore not been possible thus far to understand in detail the mechanism of antibody synthesis.

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CATABOLISM OF PLASMA PROTEIN IN THE GUT*

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Some time ago a series of experiments were carried out in which either plasma protein or albumin randomly labeled with C^{14} -amino acids was incubated with slices of rat liver or with homogenates from various tissues or with Ehrlich ascites tumor cells (from mice).¹ The results of these experiments, except those with spleen homogenates and those with the tumor cells, turned out to be entirely negative in that no breakdown of albumin was found. No C^{14} could be detected either in the respiratory carbon dioxide or in the trichloroacetic acid (TCA) soluble activity from any of the preparations after 2.5- to 5-hour incubations. These results, although they did not agree with those previously reported by Roberts and Kelley,² have been confirmed by Katz and his co-workers.³ Penn, on the other hand, has reported a requirement for ATP and CoA for breakdown of albumin in homogenates of liver and brain.⁴ Numerous other workers have concerned themselves with measuring the breakdown of albumin and other plasma proteins *in vivo* in various systems, for example in perfused livers. These results are also not in accord; consequently they will not be dealt with here.

The catalytic effect of these disagreeable results caused us to evolve a concept new to us. Generally research carried out in this area was predicated on the assumption that the circulating proteins broke down in some one tissue or tissues, if not in the blood, but the possibility exists that such is not the case, and that the protein gets into the gastrointestinal tract by some route. There it would be digested by the extracellular proteases and be reabsorbed as amino acid. Therefore, experiments have been carried out to test the possibility that part at least of the albumin involved in turnover in the normal animal is broken down in the gastrointestinal tract following its synthesis in the liver.

Methods

General procedure. Experiments of three main types have been carried out:

(1) Loops of rabbits' intestine were isolated and irrigated *in situ* with a dilute solution of albumin. Meanwhile albumin labeled with I^{131} was injected intravenously into the animals. Analyses were then made on the irrigating fluid with the object of identifying albumin- I^{131} in it.

(2) Loops of intestine from rabbits were isolated and filled with dilute albumin solution. Again albumin- I^{131} was injected intravenously. One hour later the loops were dissected out and rapidly frozen in dry ice. While still frozen the tissue was scraped off, leaving a solid sausage of contents. Analyses were made on this material.

(3) Rats were fed soybean meal and were given soy-bean trypsin inhibitor in their drinking water. They were then injected with albumin- I^{131} , and their

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feces were collected over a period of one day. Analyses for albumin- I^{131} , trichloroacetic acid (TCA) soluble and TCA-insoluble activity were made in their feces. Experiments of types 1 and 2 with rabbits have been reported elsewhere⁵ and are summarized here.

Specific methods. In the case of labeled albumin, rabbit, rat, or bovine serum albumin was iodinated by the method of Gilmore and his co-workers⁶ using iodine- I^{131} in the amount of 4 atoms per mole of albumin, but modified in that potassium persulfate (2 ml. saturated) was used in place of ammonium persulfate. The preparation was freed of iodide by dialysis against buffer containing a trace of thiosulfate and iodide for 24 hours using several changes and, finally, against water. Most preparations then contained less than 2 per cent free iodine (TCA soluble radioactivity).

Isolation and identification of albumin. Albumin was isolated from intestinal contents by precipitating it with TCA and then redissolving the precipitate in 96 per cent ethanol.^{7,8,9} The identity of the redissolved protein was confirmed by electrophoresis against known samples of pure albumin in the usual barbital buffer at pH 8.6 or, in some cases, against a phosphate buffer at pH 6.8.

Treatment of feces from rats. The 24-hour fecal matter was dried in a desiccator, ground up, and weighed. Samples of known weight were taken and placed in vials with 5 per cent TCA for counting of I^{131} to obtain the total activity. Then the residue was extracted several times with TCA until the residual radioactivity remained constant. TCA-soluble radioactivity was obtained by difference.

Other fecal samples, collected directly prior to the 24-hour collections, were dried, 100 mg. of albumin in 5 ml. of water were added as carrier, and the sample was extracted for 0.5 hour. The whole mass was precipitated with an equal volume of 10 per cent TCA; the centrifugate was washed with 1 per cent TCA, and the precipitate was extracted with 96 per cent ethanol. The ethanoic solution of albumin (about 1 ml.) was used directly as 10 to 50 microliter aliquots for zone electrophoresis on paper (Whatman No. 3). Alcoholic solutions cannot be used directly in this fashion on cellulose acetate membranes (millipore strips), which are otherwise often more convenient than paper.¹⁰

The TCA-soluble activity in the intestinal contents of the rabbits and in the feces from the rats was also examined electrophoretically. In all cases substances moving with the same mobilities as mono- and diiodotyrosine were found to be present, generally in the ratios of about 1 to 1 (moles).

Determination of radioactivity. The radiation from I^{131} was measured with a scintillation well counter (crystal) in a vial containing a standard volume of solution. For detection of radioactivity on paper, the strips were cut into short sections crosswise to the direction of electrophoresis, and the sections were placed in the bottom of the counting vials together with a standard volume of dilute base. Counting was carried out for sufficient time to establish values within an error of ± 5 per cent.

Animals and their treatment. Rabbits were starved for 24 hours and, before operating on them, they were heavily anesthetized with sodium pentobarbital after sedation with morphine. A section of the intestine about 25 cm. from the stomach was isolated by dilating a pair of small balloons about 12 cm.

apart. The section was then irrigated via two small plastic tubes at a rate of 1 ml./min. The outflow was collected directly in 20 per cent TCA. Blood samples were taken at intervals during the experiment for determination of total I^{131} . Contents of the irrigating fluid, which contained 1 per cent albumin, were tested for blood by the benzidine method. In no experiment was a significant amount found.

Experiments with rabbits in which sections of intestine were frozen involved much less trauma to the animals, especially to their intestines because the intestine was not sectioned prior to its complete removal. The section of intestine was isolated by gently encircling it with 0.7 cm. umbilical tape. Then it was filled until it was slightly distended with an albumin-containing solution injected through a 27-gauge needle. Control experiments were carried out in which rabbits were injected with iodide- I^{131} instead of albumin- I^{131} .

The rats used were of the Sprague-Dawley strain, and were kept in metabolic cages so that the feces could be collected on paper towels. In the cases where the fecal products were examined electrophoretically to establish the fact that albumin was present, they were collected individually and directly from the rats, prior to making the 24-hour collection. The period of the collection, following the intraperitoneal injection of the albumin- I^{131} , was determined by the appearance in the stool of a marker fed to the animals at the beginning.

All animals whose feces were collected were maintained on a soy-bean meal diet; in addition, the experimental groups were given soy-bean meal trypsin inhibitor (Mann) in their drinking water at the rate of 10 mg. per ml., as well as 3 ml. of the same inhibitor-containing solution prior to the start of the experiment.

Results and Discussion

The results, shown in part in TABLE 1, demonstrate that in rabbits in which sections of the intestine were irrigated, labeled albumin appears in the contents from the section. Identification of the albumin rests on the two unique properties: solubility in alcoholic-TCA^{7,8,9} and movement in electrophoresis at pH 8.6 at the same rate as a known sample of albumin. Similar results were obtained with rabbits in which sections of the intestine were frozen (TABLE 2). Analysis of the fecal contents from rats given trypsin inhibitor also showed the presence of a protein-like material with the properties of albumin (TABLE 3). From these results it must be concluded that, at least qualitatively, some albumin is transferred into the gastrointestinal tract in the two species under surveillance.

The appearance of other large polymers in the feces or gut following their injection into the blood circulation has been shown in the case of human subjects given polyvinyl pyrrolidone (PVP, molecular weight about 40,000) labeled with iodine^{11,12} and, in mice, given dextran labeled with tritium.¹³ In addition it has been shown that rats whose intestines have been irradiated with X rays excrete intravenously injected PVP- I^{131} in the feces in large amounts.¹⁴

The route or routes by which the protein gets into the tract under normal conditions is less clear. However, the following pertinent observations have

TABLE 1
APPEARANCE OF RADIOACTIVE IODINE IN FLUID IRRIGATING RABBIT
INTESTINES AFTER ALBUMIN- I^{131} *

Rabbit	Period* (min.)	Fraction excreted per hour ($\times 10^6$)	
		Total	TCA-insol.
7	239	43	27
10	400	45	30

Average radioactivity levels in whole blood: R 7, 1.06×10^6 cpm per ml.; R 10, 1.18×10^6 cpm per ml.

* Following intravenous injection of labeled albumin containing 4.6 per cent (R 7), and 1.2 per cent (R 10) free iodide- I^{131} . In rabbit 9, which was given iodide- I^{131} , the irrigating fluid was found to contain only TCA-soluble activity.

TABLE 2
DISTRIBUTION OF RADIOACTIVE IODINE IN INTESTINAL CONTENTS*

Rabbit	6	8	4	5
TCA-soluble	78	85	77	78
TCA-insoluble	22	15	23	22
Origin			5.9	3.5
Albumin			9.0	8.9

* Contents from frozen sections of intestine. In rabbit 7, given iodide- I^{131} , none of the activity was TCA-insoluble.

TABLE 3
DISTRIBUTION OF RADIOACTIVE IODINE IN SAMPLES OF FECES*

Rat group	A	B	C
	per cent		
TCA-soluble	53	48	45
TCA-insoluble	47	52	55
Origin	27	31	35
Albumin	9	14	13

* Feces uncontaminated with urine from rats fed soybean trypsin inhibitor.

been made: protein with the properties of albumin appears in the gastric secretions of normal human subjects¹⁵ and in the bile in many different species.¹⁶ Moreover, a protein with the properties of albumin appears to be present in the pancreatic juice obtained from rats.

Considerable experimentation has also been carried out with human subjects, and this has led to the identification of hypoalbuminemia associated with massive hypertrophy of the gastric mucosa¹⁷ or with intestinal lesions.¹⁸ In such conditions hypercatabolism of albumin occurs, presumably due to excessive loss of the protein into the gastrointestinal tract, and the turnover of albumin and γ globulin is increased (pool size decreased, and the rate of

catabolism increased). This type of pathology has recently been reviewed by Holman and his co-workers;¹⁹ however, the existence of hypercatabolic hypoproteinemia was first deduced by Albright and his co-workers.²⁰ The high turnover rate of albumin in two cases was investigated by Schwartz and Thomsen.²¹ Loss of albumin into the stomach or intestine associated with a high turnover rate and hypoalbuminemia has been observed by Citrin and co-workers,¹⁷ and by Steinfield and his co-workers.¹⁸

Thus it appears that in the normal animal and in human subjects, particularly those with pathological conditions of the gastric or intestinal mucosa, it can be demonstrated that albumin and other serum proteins are lost into the gastrointestinal tract. However, the quantitative measurement of this loss, and its relation to the normal turnover of these proteins is much more difficult to evaluate.

TABLE 4
CALCULATION OF ALBUMIN CATABOLIZED BY SLICES FROM RAT-LIVER*
Fifteen-mg. Albumin Incubated with 150-mg. Slices for 5 Hours in 3-ml. Krebs-Ringer Phosphate Medium

	A	B
From C ¹⁴ O ₂ production	5.7	329
From glycogen formation	0.93	54
From glucose formation	3.65	211
Total		594

A: Per cent per 5 hr. per 15-mg. slices.

B: Milligrams per day per 12-gm. liver (assuming a 200-gm. rat has a 12-gm. liver).

* Roberts and Kelley,² Table II, p. 562 (from this table it is not quite clear whether the C¹⁴O₂ production is for a 2.25- or a 5-hour period. It has been assumed that it was calculated to a 5-hour basis).

The turnover data of Jeffay and Winzler,²² for example, obtained with normal rats show that this species catabolizes albumin at a rate of 91, 114, or 235 mg. per day (when calculated to the basis of a 200-gm. rat) when the diet of the animals contains 0, 12, or 30 per cent casein respectively. When the catabolism of albumin-I¹³¹ by the perfused liver from rats (about 300 gm.) was examined by Gordan²³ the rate was found to be 1 mg./hour. Thus the liver catabolized albumin at 14 per cent of the rate indicated by the turnover experiments on the intact animal on a 12 per cent casein diet. Unfortunately, the data from these perfusion experiments and, from the experiments of Armstrong and Tarver¹ and of Katz and his co-workers,³ carried out with tissue slices or homogenates of liver, are in conflict with those of Roberts and Kelley.² The data obtained by these workers are shown in TABLE 4.

These results are excessively high, a conclusion supported by Katz and his co-workers.³ It appears necessary to assume that the catabolism of albumin by the rat liver cannot account for more than about 25 per cent of the catabolism that must be postulated to exist to account for the turnover breakdown. Seventy-five per cent of the catabolism is unaccounted for,²⁴ since no other tissue appears to catabolize albumin at a high rate.

TABLE 5
FECAL EXCRETION OF RADIOACTIVITY
Following Injection of Albumin- I^{131} into Sprague Dawley Rats, Group A

	Control	Experimental
Weight (gm.)	178 (5)*	162 (6)
Feces (gm.)	2.8	2.3
Serum albumin (mg./ml.)	24	23
Activity in blood (cpm/ml. $\times 10^{-5}$)	5.3	5.3
Total excreted (cpm $\times 10^{-6}$)	0.36	1.3
TCA insol. excreted (cpm $\times 10^{-6}$)	0.022	0.69
Excretion rate (mg./day)	1.0	29

* Number of animals in brackets.

TABLE 6
FECAL EXCRETION OF RADIOACTIVITY
Following Injection of Albumin- I^{131} into Rats

	Weight (gm.)	Excretion rate (mg./day)
Group A Control	178 (5)*	1.0
Experimental	162 (6)	29
Group B Control	136 (6)	0.4
Experimental	131 (6)	20
Group C Control	142 (7)	0.5
Experimental	140 (6)	17

All animals on diet containing soybean meal. Experimental animals given trypsin inhibitor.

* Number of animals in brackets.

TABLE 7
CALCULATED LOSS OF ALBUMIN INTO SECTIONS OF INTESTINE*

	Rabbit Nos.	
	7	10
(1) Weight (kg.)	3.40	4.54
(2) Dose, albumin- I^{131} (cpm $\times 10^{-8}$)	1.02	3.6
(3) TCA-insol. Radioactivity lost (cpm per hr. $\times 10^{-4}$)	2.75	1.08
(4) Radioactivity in blood (average) (cpm per ml. $\times 10^{-6}$)	1.06	1.18
(5) Total albumin ² (g)†	10.2	13.5
(6) Radioactivity in blood albumin‡ (from (4) and (5) cpm per mg. $\times 10^{-4}$)	2.12	2.38
(7) Albumin lost (mg. per hr.) (3) \div (6)	1.3	0.45

* Loss into 12-cm. section of intestine (rabbit).

† Calculated assuming blood volume is 6 per cent body weight, 2 per cent albumin in whole blood, and total albumin = circulating albumin $\times 2.5$.

‡ Average value for experimental period.

The fecal excretion of albumin found in the present experiments (TABLE 5), which must represent a minimum estimate of albumin excretion into the gastrointestinal tract, was calculated to be between 17 and 29 mg. per day, on the assumption that TCA-insoluble material was all albumin or derived from albumin (TABLE 6). This represents about 15 to 25 per cent of the loss which must be postulated in order to account for the normal turnover for rats on a 12 per cent casein diet.²² However, it is likely that much of the TCA-soluble I^{131} found in the fecal contents from the rats also arose from albumin breakdown (and not from the free iodide- I^{131} in the albumin preparations). Hence gastrointestinal breakdown may account for a greater per cent of the turnover breakdown than that calculated from the TCA-insoluble activity found in the feces and, in fact, may account for most of the extrahepatic breakdown.

The relationship between turnover breakdown and intestinal breakdown in rabbits is still less clear since data for loss of albumin into short sections of parts of the intestine cannot well be extrapolated to loss into the intact and complete gastrointestinal tract. However, in TABLE 7 approximate calculations are made of the rate of loss of albumin into 12-cm. sections of intestine in the case of rabbits 7 and 10. Since, many assumptions are made in order to arrive at the final figures of 1.3 and 0.45 mg./hour, little emphasis can be placed on these figures, although they do indicate that, if the rest of the intestine did behave like the sections under consideration, the total loss into the gut would have accounted for a very significant fraction of the turnover breakdown.

Summary

It has been shown in experiments with normal rabbits and rats that a radioactive protein with the properties of serum albumin appears in the intestine (rabbits) or feces (rats) when the labeled protein is injected into the animals. The significance of these results is discussed with respect to the normal turnover of the plasma proteins and with respect to the loss of protein which occurs in some pathological states which have been observed in human subjects. The tentative conclusion is reached that a large fraction of the normal turnover breakdown of albumin in animals may be accounted for by loss and breakdown in the gastrointestinal tract.

Acknowledgments

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THE AMINO ACID INTERRELATIONSHIPS BETWEEN THE VARIOUS SERUM PROTEINS OBTAINED BY SALTING OUT, ELECTROPHORESIS, AND COLUMN CHROMATOGRAPHY*

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Although the title of this monograph is *Plasma Proteins in Health and Disease*, the studies that we shall present are limited to the serum proteins. Most of the evidence to date (Block, 1935; Davis, private communication) indicates that fibrinogen should be considered to be a separate constituent of the blood, although Schultz *et al.* (1955) have suggested from its amino acid pattern that fibrinogen belongs to the family of serum proteins.

Until the middle of the 19th century, it was believed that blood serum was composed of a single protein called albumen. In 1878 a French pharmacist, C. J. Méhu, was the first to show that proteins could be precipitated from urine, apparently without change in their physical properties, when the urine was saturated with ammonium sulfate. Nine years later, Lewith (1887) used ammonium sulfate to fractionate sera and reported the presence of two proteins later designated globulin and albumin. Following Lewith's work, the use of neutral salts to separate serum proteins into albumin and globulin was widely employed as an aid in clinical diagnosis, for it was found that the ratio of albumin to globulin (A/G) in disease differed from that found in the blood of healthy individuals.

It appears that because the A/G ratio was of clinical value, the idea that there were but two distinct proteins in serum was generally accepted. This was true even though various investigators including Hardy (1905), Sørensen (1930), Gróh and Faltin (1931), and Block (1933, 1934) presented evidence that the proteins isolated from serum by different methods appeared to be the result of the method of fractionation employed. These investigators suggested that serum was not composed of just albumin and globulin, but that it contained a great number of interrelated proteins. Hardy, from the results of experiments on the behavior of serum in an electrical field (electrophoresis), suggested that serum is not a mixture of independent proteins, but that it is composed of a complex protein that breaks down readily into fractions, the composition and properties of which depend upon the degree of dilution and the reagents used. Hardy's ideas were supported by the physicochemical studies of Sørensen and the amino acid analyses of Gróh and Faltin and of Block. In order to emphasize the concept that serum may be composed of a multitude of related polypeptides, Block (1933, 1934) proposed that the term orosin be used to designate the total coagulatable protein of serum from which various homogeneous proteins could be derived by physical and chemical manipulations.

What is the experimental evidence for this suggestion? In addition to Hardy's work just mentioned, Sørensen showed that lipid-free crystalline serum albumin is easily and reversibly dissociated into components of different

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physical and chemical properties. Block (1933, 1935) found that the lysine content of the globulins prepared by salting out serum with magnesium sulfate, sodium sulfate, ammonium sulfate, sodium chloride was variable, although the quantity of arginine remained quite constant. Similar discrepancies in the lysine to arginine ratio were also noted in the albumins. On the other hand, when orosin from normal and nephrotic human sera was analyzed for arginine and lysine, it was found that in spite of the large differences in the A/G ratios between the normal and nephrotic sera, the ratio of arginine to lysine was the same as that found in the blood from healthy individuals.

To summarize the situation in 1937, serum was generally considered to be composed of 2 discrete proteins, albumin and globulin, even though a few individuals had suggested otherwise. In that year, Tiselius introduced his new apparatus for the electrophoretic analysis of serum proteins. As a result of this invention and the more recently introduced methods of paper and starch electrophoresis, I think that most people now believe that there are at least 5 or 6 discrete proteins in serum: albumin, α_1 -globulin, α_2 -globulin, β -globulin, and probably several γ -globulins and a few minor components. These electrophoretically separated serum protein fractions are generally believed to be independent variables in the serum and to be unrelated to each other. Some investigators are, however, beginning to doubt the complete independence of the various electrophoretically separated components. Thus Brackenridge (1960) reported that the amounts of α_1 -globulin, α_2 -globulin and β -globulin are highly interrelated in sera from normal individuals. On the other hand, the quantities of γ -globulins show a much larger variation from person to person (Jeffay and Winzler, 1958). Brackenridge (1960) explains these differences on the basis that the γ -globulins are not made in the liver. Strickland *et al.* (1959) also doubt the value of the classification of the serum proteins based upon electrophoretic methods. They state "This work underscores the fact that the division of serum proteins into 5 fractions is purely arbitrary and essentially meaningless."

Although a number of investigators have used various kinds of column chromatography in an attempt to fractionate the serum proteins, the most successful approach has been that devised by Peterson and Sober in 1956 in which anion- and cation-exchange cellulose are employed. Chromatography on ion-exchange cellulose results in the fractionation of the serum proteins into many discrete peaks. In fact the number of peaks obtained from the cellulose column appears to be limited only by the types of eluting agents used and the desires of the investigator. Some of the protein fractions obtained by this method (Sober *et al.*, 1956; Peterson and Sober, 1956) are electrophoretically homogeneous; however, the majority is not.

Thus, in the last 100 years, the ideas concerning the number of distinct proteins present in serum have changed from a single protein (albumen), to 2 proteins (albumin and globulin), to 5 or more electrophoretically homogeneous proteins and, presently, to the idea of very many discrete proteins. Is there any validity then to the earlier suggestion that serum contains a giant molecule, orosin, which in turn is composed of many interrelated proteins and that the properties of the protein fractions isolated from the serum are dependent, in part at least, on the reagents used?

The earlier conclusions were and could be criticized on the basis of inadequate methodology. Evidence will now be presented that appears to support the hypothesis that whatever method is used for fractionating the serum proteins the ratio of certain pairs of amino acids remains relatively constant in spite of large differences in their amount per unit of nitrogen.

TABLE 1 shows the electrophoretic composition of the protein fractions precipitated from bovine serum by step-wise increases in the quantity of ammonium sulfate (Keller and Block, 1958a). The ammonium sulfate was introduced into the aqueous solution of the serum proteins by dialysis of the serum against salt solutions of increasing strength according to Cohn *et al.* (1940). It should be noted that even at 50 per cent saturation of ammonium sulfate, the precipitate contains some protein that behaves as does albumin in paper electrophoresis.

TABLE 1
ELECTROPHORETIC COMPOSITION OF HUMAN SERUM PROTEIN FRACTIONS OBTAINED FOLLOWING FRACTIONATION WITH AMMONIUM SULPHATE

Fraction	Ammonium sulfate as per cent of saturation	Per cent nitrogen*	Protein components in per cent of total			
			γ -globulins	β -globulins	α -globulins	Albumins
A	28	12.5	80.4	19.6	0	0
B	35	13.4	72.2	27.8	0	0
C	42	12.4	52.2	24.3	23.5	0
D	50	10.5	20.7	37.7	23.2	18.4
E	57	12.2	0	25.1	38.8	36.1
F	65	12.1	0	22.0	27.2	50.8
G	72	8.7	0	0	0	100.0

* Uncorrected for moisture and ash.

Each of these protein fractions was then analyzed for 12 amino acids by paper chromatography (Block *et al.*, 1958). In order to increase the comparative aspects of the analyses, all determinations for any group of amino acids were carried out simultaneously.

FIGURE 1 shows that in spite of variations of two- or threefold in the absolute quantities of certain amino acids per unit of nitrogen, the ratios of the pairs, aspartic acid:lysine and glycine:serine, remain quite constant for all or almost all the protein fractions. On the other hand, this chart illustrates the fact that some amino acids, for example methionine, could not be paired with any other amino acid for which analyses were made.

Schultz *et al.* (1955) used another technique for separating the serum proteins, namely starch electrophoresis. They found that the ratio of the pair, glutamic acid:alanine remained approximately constant not only among the 5 or 6 electrophoretically separated proteins, but also in the sera from 3 mammals: dog, rat, and human. This can be seen on FIGURE 2, which is taken from the paper of Schultz *et al.* The data also show that the ratio of threonine:aspartic acid is constant for all fractions except the γ -globulins, that alanine and glycine show a reciprocal relationship calculated per 100 mols of aspartic acid, and that the ratio of the sum of glycine plus alanine:aspartic acid is relatively constant.

If the electrophoretically homogeneous serum protein fractions—albumins, α_1 -globulin, α_2 -globulin, β_1 -globulin, β_2 -globulin, and 2 γ -globulins—are heated with 0.03 *N* HCl at 105° C., aspartic acid is split out of the peptide chain, according to Schultz and Delavan (1959). The components in the hydrolyzates can then be separated on 2-dimensional chromatograms using high voltage paper electrophoresis in one direction and paper chromatography in the second. FIGURE 3 shows the results of these treatments. The heaviest spot is aspartic acid,

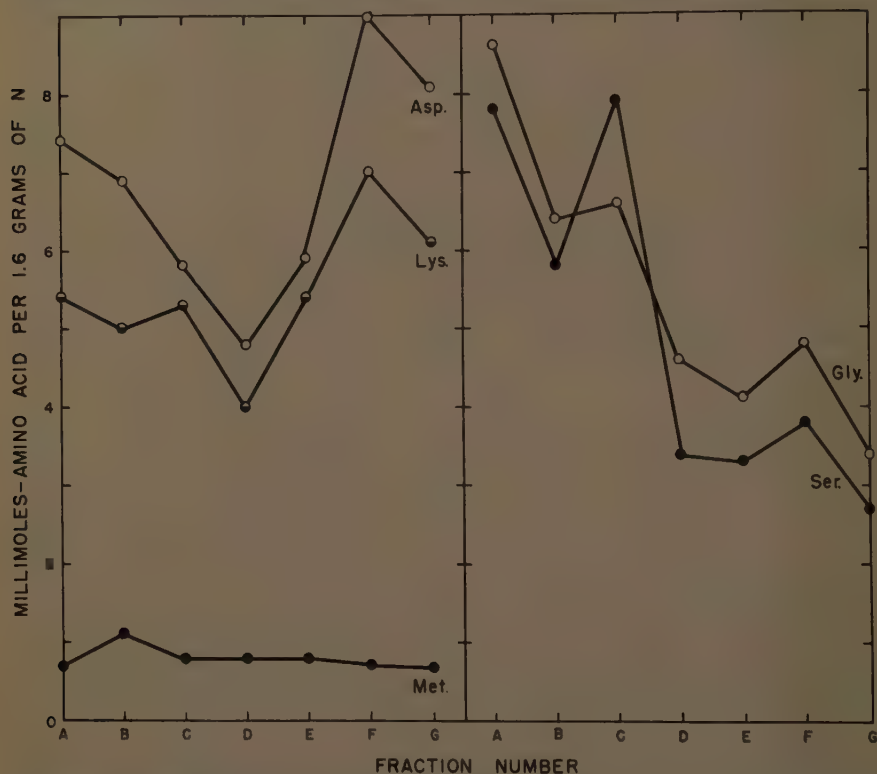


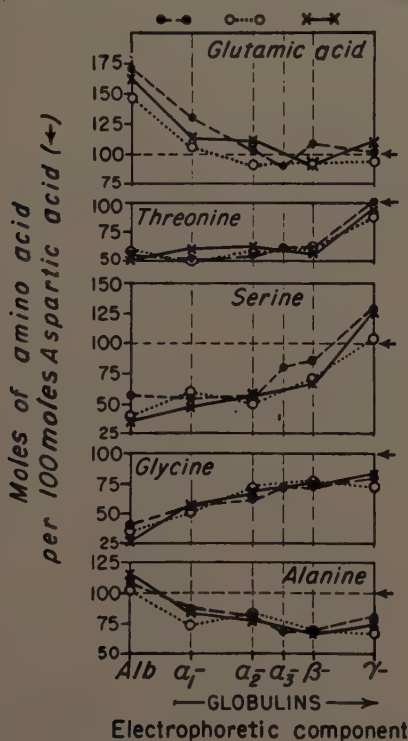
FIGURE 1. Related and unrelated amino acids in human serum protein fractions prepared by fractional precipitation with ammonium sulfate.

the other spots are due to peptides. It is apparent that the member and location of the peptides, present in largest amounts, are the same in the hydrolyzates prepared from each of the serum protein fractions. This is the case even though the absolute quantity of any peptide (or possibly a mixture of similar peptides) varies from fraction to fraction.

Thus the results obtained from protein fractions prepared by salting out with ammonium sulfate and by starch electrophoresis followed by partial or complete hydrolysis are in accord with the suggestion that the serum proteins contain certain groups of amino acids in constant ratio.

In order to approach this problem in another way, human serum proteins were fractionated (Keller and Block, 1958b) on a column of diethylaminoethyl-

cellulose (Sober *et al.*, 1956; Peterson and Sober, 1956). TABLE 2 shows the nitrogen content and the percentage distribution of the various electrophoretic components in these fractions.* The wide variations in the nitrogen content should be noted. It appears from these results that one half or more of many of the γ_1 - and β -globulins is nonprotein material. Note also the electrophoretic heterogeneity of some of the fractions. The amino acid compositions of nine



The values for gram moles of amino acid per 100 moles aspartic acid are plotted against the major protein groups of dog, rat, and human sera, arranged in order of decreasing mobility.

FIGURE 2. Related amino acids from human, dog, and rat serum protein fractions separated by electrophoresis (from Schultz *et al.*, 1955).

of these fractions (A, D, F, G, H, J, K, M, P) were estimated by paper chromatography (Block *et al.*, 1958). Six pairs of amino acids were found to have a relatively constant molar ratio of all nine protein fractions. An example of one such pair is shown on FIGURE 4. It will be seen that although the absolute quantity of the amino acids per unit of nitrogen varied as much as threefold, the ratio of the two pairs of amino acids remained relatively constant in all the fractions. This is more interesting when we remember that some of these

* Fahey and Horbett (1959) have confirmed our finding (Keller and Block, 1958b) that DEAE cellulose resolves the γ -globulins into at least five fractions.

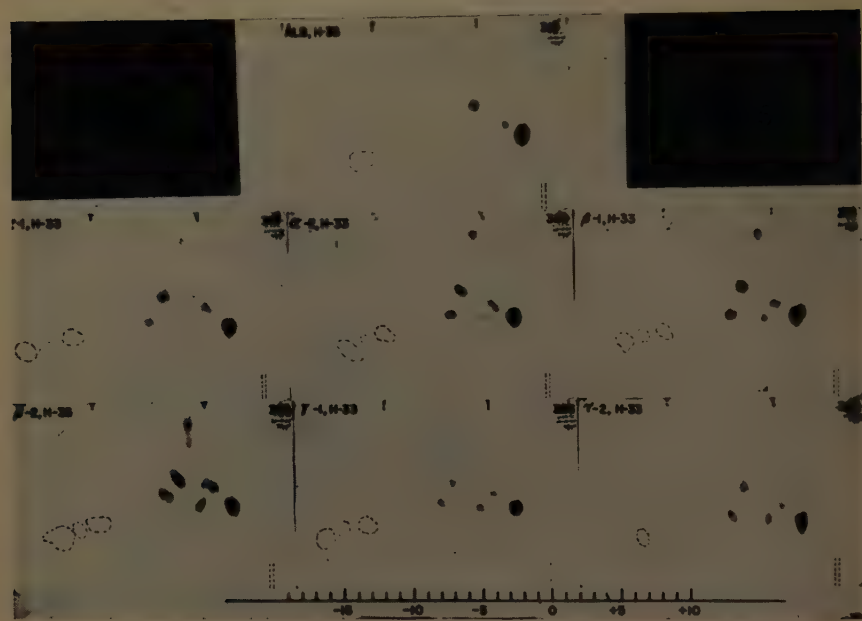


FIGURE 3. Two-dimensional chromatograms of hydrolyzates from electrophoretically homogeneous human serum protein fractions (Schultz and Delavan, 1959).

TABLE 2
ELECTROPHORETIC COMPOSITION OF HUMAN SERUM PROTEIN FRACTIONS
SEPARATED ON DEAE CELLULOSE

Fraction	Per cent nitrogen*	Components (per cent of each fraction)						
		γ_2	γ_1	β_2	β_1	α_2	α_1	Albumins
A	13.5	100						
B	12.6		100					
C	9.4		100					
D	9.0		100					
E	8.6		100					
F	5.9		100					
G	6.7				100			
H	5.4				100			
I	8.4			100				
J	9.8			66.4		33.6		
K	15.2			10.1				89.9
L	15.0					11.2	14.7	74.1
M	15.0			4.8		13.6	13.3	68.3
N	13.8			12.1		27.1	16.7	44.1
P	12.0					57.3		42.7

* Uncorrected for moisture and ash.

fractions are electrophoretically homogeneous (A, D, F, G, H) while others are mixtures of β - and α -globulins or of albumins and α -globulins.

It is recognized that paper chromatography of certain amino acids may be subject to rather large analytical errors (Block *et al.*, 1958); consequently the doubt arose as to whether the constant amino acid ratios were simply analytical

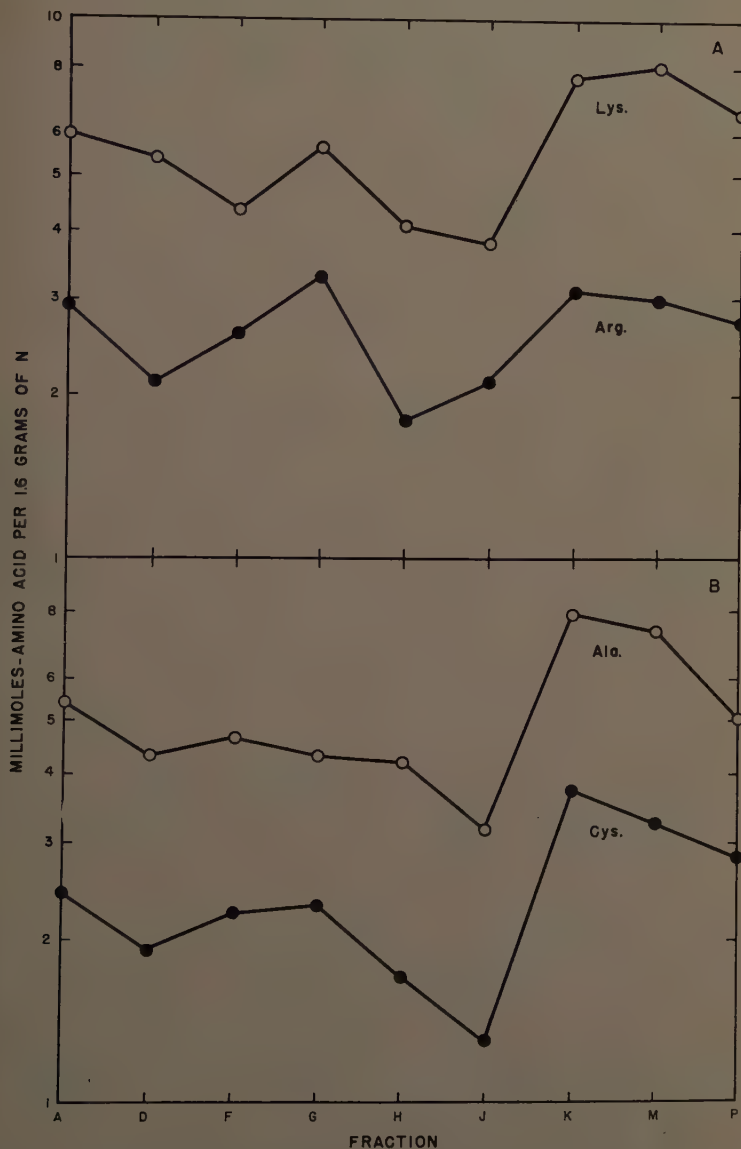


FIGURE 4. Related amino acids in human serum protein fractions prepared by chromatography on DEAE cellulose.

artifacts. Therefore, 14 fractions (A, B, C, E, F, G, H, I, J, K₁, K, L, N, P) have been recently analyzed using the accurate ion-exchange method of Moore and Stein (1951, 1954), but employing the Technicon Autoanalyzer and the varigrad (Peterson and Sober, 1959) for the gradient elution of the amino acids according to Piez and Morris (1961). The analytical results obtained by this method are given in TABLE 3. These data confirm the previous conclusions, namely that in spite of marked variations in the absolute amounts of an amino acid per unit of nitrogen, the molar ratios of certain pairs remain relatively constant. These relationships are further illustrated on FIGURE 5. Fourteen

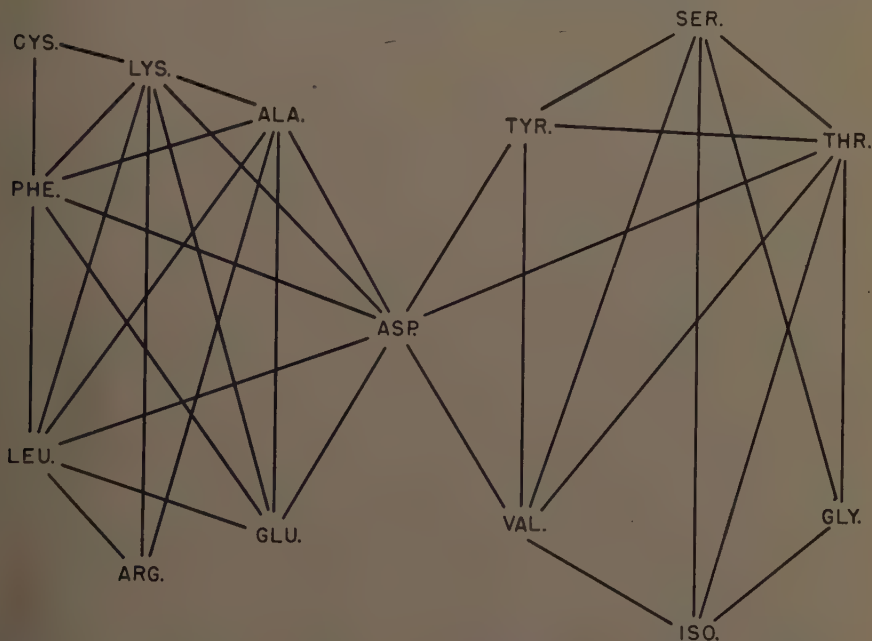


FIGURE 5. Interrelated amino acids in human serum protein fractions prepared by chromatography on DEAE cellulose. All interconnected amino acids are related at the one per cent level of rank analysis and at 10 per cent deviation from the mean molar ratios. The arrangement of the individual amino acids on the diagram is purely arbitrary.

of the 18 amino acids, for which analyses were made, have relatively constant molar ratios with 2 or more amino acids at the 1 per cent level by rank analysis and at less than 10 per cent deviation from the mean molar ratios. One pair of amino acids (histidine and methionine) is related to each other, but not to any other amino acid. Proline does not appear to be related to any other amino acid when tested by the criteria mentioned above. Hydroxyproline, found in only 2 of the 14 fractions, is obviously an unrelated amino acid.* The results given in TABLE 3 show that, except for hydroxyproline, the amino acid composition of the γ_2 -globulin (fraction A) is almost the same as that of the

* Tryptophan, for which analyses were not made, would also be an unrelated amino acid as it has been shown not to be present in purified human albumin (unpublished results obtained from A. Saifer, Isaac Albert Research Institute, Brooklyn, N. Y.).

γ_1 -globulin (fraction E), but that the amino acid patterns in 2 of the γ_1 -globulins (fractions E and F) differ significantly. The amino acid compositions of the 2 β_1 -globulins (fractions G and H) are also practically the same except for the absence of hydroxyproline in fraction G and the strikingly high quantity in fraction H. In fact, to our knowledge, this β_1 -globulin (fraction G) is the only protein in which the quantity of hydroxyproline exceeds that of any other amino acid by a factor of two.* In this connection, it is interesting that although Waldschmidt-Leitz *et al.* (1960), using paper chromatographic methods, have reported that human γ -globulin contained 0.85 per cent of the total *N* as hydroxylysine *N*, no evidence for this amino acid was found in any of our fractions. If this discrepancy is not due to the different methods used, it may indicate that the composition of the serum proteins is influenced by genetic (Earle *et al.*, 1959) or dietary factors.

The values for cystine and methionine given in TABLE 3 are calculated from the sum of cystine plus cysteic acid and of methionine plus methionine sulfoxide in those cases where the oxidation products were present. It should be noted that in three instances (fractions E, F, and P) only cysteic acid and no cystine was found. At first glance it could be assumed that the cystine was oxidized during or after the preparation of the protein; if this were the case one would also assume some oxidation of methionine. However, in the case of fractions F and P, there was no evidence of methionine sulfoxide. This raises the interesting question whether cysteic acid may be a normal constituent of some proteins or whether certain amino acid residues adjoining the cysteine rests predispose them to oxidation during hydrolysis.

Chicken (Block *et al.*, 1959) and turtle (Block and Keller, 1960) sera were also fractionated on DEAE cellulose, and the protein fractions were analyzed by paper electrophoresis and paper chromatography. The amino acid values were subjected to statistical analysis. The interrelationships that were found in the chicken serum proteins are shown graphically on FIGURE 6. The molar ratios of 6 pairs of amino acids in all 11 fractions were significantly constant at the 1 per cent level. This is indicated by the double lines on the chart. Those amino acids that were found to be related to one or more at the 5 per cent level of significance are linked by single lines. In all, of 105 correlations, 6 pairs (valine:glycine; valine:cystine; lysine:phenylalanine; arginine:threonine; arginine:tyrosine; and aspartic acid:serine) were found to be related at the 1 per cent level, and 26 pairs at the 5 per cent level of significance. If these interrelationships were due solely to chance, one would expect to find 1 or 2 pairs at the 1 per cent level and 5 or 6 pairs at the 5 per cent level. In the case of the turtle serum proteins, 12 pairs of amino acids were found to have significantly constant molar ratios at the 1 per cent level, and 24 pairs at the 5 per cent level of 105 possible combinations. When this type of statistical evaluation of the data was employed, each amino acid for which an analysis was made could be paired with at least one other amino acid except for glutamic acid in the chicken serum and serine in the turtle serum proteins.

Because of the high degree of correlation between the different amino acids

* Sycamore cell-wall proteins have been reported to yield 15 per cent hydroxyproline, 5.3 per cent proline, 0.0 per cent hydroxylysine, and 15 per cent lysine (Lamport and Northcote, 1960).

that was found by the statistical methods, it was thought advisable to carry out the same type of statistical treatment on 8 apparently unrelated proteins. Therefore the statistician was given the amino acid composition of bovine serum albumin, human hemoglobin, crystalline egg albumin, insulin, β -lactoglobulin, actin, gelatin, and ribonuclease. The data were presented to him as the amino acids in fractions 1 to 8, inclusive. The statistician reported that of 105 pairs, 5 pairs were significantly related at the 5 per cent level and that 3 pairs had a significant negative correlation. This distribution is that expected

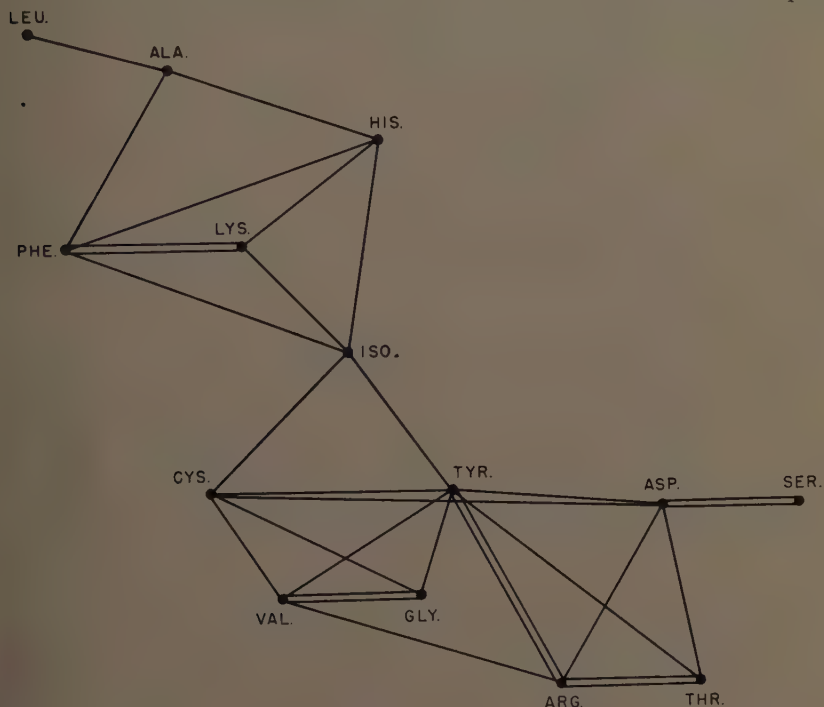


FIGURE 6. Interrelated amino acids in chicken serum protein fractions prepared by chromatography on DEAE cellulose (double lines indicate 1 per cent significance, single lines indicate 5 per cent significance). The arrangement of the individual amino acids on the diagram is purely arbitrary.

by chance. In contrast the statistical treatment of the results obtained on the chicken serum fractions, in which there were also 105 pairs of amino acids possible, 26 pairs were correlated at the 5 per cent level of significance. There were no negative correlations.

The studies reported here may be summarized as follows:

(1) Mammalian, avian, and reptilian sera were fractionated by various methods and the resulting protein components were characterized by paper electrophoresis and analyzed for their constituent amino acids.

(2) The results showed that the molar ratios of a number of pairs of amino acids remained constant in all the serum protein fractions although their absolute quantities per unit of nitrogen varied markedly. These findings

suggest that all the serum proteins are composed of amino acid *anlagen*, that is, a limited number of different peptides, each of which has a relatively constant amino acid content, but differs from the others in amino acid composition.

(3) These data are also interpreted to mean that serum is not composed of a large number of discrete, unrelated proteins, but that the serum proteins are interrelated and that the isolated components are the result of the method of fractionation employed. That is, the currently described experimental results appear to support the concept that serum is not a mixture of various unrelated proteins, but the protein fractions, whether homogeneous or not, are interrelated and may exist in the living tissue as a complex, easily dissociable molecule that has been called orosin (Block, 1934).

It should be stressed that the *anlagen* and orosin hypotheses do not in any way preclude the possibility of isolating, by physicochemical methods, completely homogeneous proteins from the serum. Thus, although bovine mercaptalbumin can be fractionated into a number of components on DEAE cellulose (Keller and Block, 1959), these fractions appeared to have the same amino acid pattern within the experimental error. In fact, Hunter and McDuffie (1959) have presented evidence that one of the human serum albumins is a continuous peptide chain.

The suggestion that the protein fractions obtained from serum by various methods (such as salting out, column chromatography, and electrophoresis) do not exist in the serum as independent proteins but are the result, at least in part, of the methods of fractionation employed is supported by the analytical data (that is, amino acid *anlagen*); moreover this idea appears to be in line with experimental evidence derived from other studies. A few of the more recent publications that may be interpreted in the light of the *anlagen* hypothesis are:

(1) Immune sera prepared against a protein preparation from tobacco (*Nicotiana debnevi*) cross react with proteins from plants representative of the major divisions of the plant kingdom (Dorner *et al.*, 1958).

(2) Biologically active proteins with quite different physiological action differ by only a few amino acids (Geschwind *et al.*, 1956; White and Landmann, 1955; du Vigneaud, 1952; and others).

(3) Proteins with essentially the same physiological action, for instance insulin, show small differences in their amino acid composition (Nicol and Smith, 1960).

Acknowledgments

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SERUM GLYCOPROTEINS: THEIR ORIGIN AND SIGNIFICANCE

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The significance of the changes that occur in the serum glycoproteins is fundamentally related to their origin. If a clear concept of the origin of these compounds existed, the question of their significance could more readily be answered. Basically, the origin of glycoproteins may be resolved into three facets: biosynthesis of the protein constituents, biosynthesis of the carbohydrate constituents, and incorporation of the carbohydrate constituents into the protein molecule. The first facet is essentially that of the biosynthesis of plasma proteins and is discussed elsewhere. However, phases of the other two facets will be considered in the following discussion.

Studies of the protein-bound carbohydrates of serum date back to the beginning of this century. The early workers noted the increase in reducing power of serum or serum proteins after hydrolysis and explained this increase as due to glucose bound to protein. Sometime later when the carbohydrate constituents were identified, glucose was not found to be among them; the major monosaccharide components of serum glycoproteins are now known to be galactose, mannose, glucosamine, and sialic acid (TABLE 1). Galactosamine and fucose are minor constituents of some serum glycoproteins. TABLE 2 presents the quantitative distribution of these constituents in serum glycoproteins.

It is well at this point to define the term glycoprotein. Most simply, the glycoproteins may be defined as compounds of the protein molecule with a substance or substances containing a carbohydrate other than nucleic acid.¹ The term is usually restricted to proteins containing 1 per cent or more of carbohydrates. By this definition all of the serum globulin fractions with high carbohydrate contents contain one or more glycoproteins. A number of proteins containing relatively large amounts of bound carbohydrate have been isolated in more or less purified form from the serum globulins. Included in this group are orosomucoid (or α_1 acid seromucoid), α_1 -glycoprotein, ceruloplasmin, haptoglobulins, α_2 -macroglobulins, prothrombin, and γ -globulin (19S fraction). In addition, there are a number of other plasma fractions with appreciable carbohydrate content that are not as well defined as the above. Some of these, for example, thyroxine-binding protein, gonadotropins, lipemic clearing factor inhibitor, hyaluronidase inhibitor, and plasma cholinesterase have important physiological functions.

Site of the Formation of Serum Glycoproteins

With the exception of some of the protein hormones, the site or sites of formation of serum glycoproteins are not definitely known. One may make the reasonable assumption that the carbohydrate moieties are incorporated into serum proteins at the same time that the polypeptide portions are formed. Some tentative concepts concerning the sites of formation of serum glycoproteins may then be inferred from information about serum globulin.

Data obtained from experimental animals in liver poisoning,² and with Eck fistulas³ indicate a lowered ability to make serum glycoprotein. A decrease has been noted in the seromucoid fraction in patients with parenchymatous liver disease.⁴ More direct evidence is provided by Miller and Bale,⁵ who reported that isolated perfused rat liver incorporated radioactively labeled lysine into plasma albumin, α -globulin, β -globulin, and fibrinogen. On the other hand,

TABLE 1

$ \begin{array}{c} \text{CHO} \\ \\ \text{HCOH} \\ \\ \text{HOCH} \\ \\ \text{HOC}-\text{H} \\ \\ \text{HC}-\text{OH} \\ \\ \text{CH}_2\text{OH} \\ \text{D-Galactose} \end{array} $	$ \begin{array}{c} \text{CHO} \\ \\ \text{HCNH}_2 \\ \\ \text{HOCH} \\ \\ \text{HOCH} \\ \\ \text{HC}-\text{OH} \\ \\ \text{CH}_2\text{OH} \\ \text{D-Galactosamine} \end{array} $	$ \begin{array}{c} \text{CHO} \\ \\ \text{HOCH} \\ \\ \text{HCOH} \\ \\ \text{HCOH} \\ \\ \text{HOCH} \\ \\ \text{CH}_3 \\ \text{L-Fucose} \end{array} $
$ \begin{array}{c} \text{CHO} \\ \\ \text{HOCH} \\ \\ \text{HOCH} \\ \\ \text{HC}-\text{OH} \\ \\ \text{HC}-\text{OH} \\ \\ \text{CH}_2\text{OH} \\ \text{D-Mannose} \end{array} $	$ \begin{array}{c} \text{COOH} \\ \\ \text{C}=\text{O} \\ \\ \text{CH}_2 \\ \\ \text{CHOH} \\ \\ \text{H}_2\text{N}-\text{CH} \\ \\ \text{HOCH} \\ \\ \text{HCOH} \\ \\ \text{HCOH} \\ \\ \text{CH}_2\text{OH} \\ \text{Neuraminic acid} \end{array} $	$ \begin{array}{c} \text{CHO} \\ \\ \text{HCNH}_2 \\ \\ \text{HOCH} \\ \\ \text{HCOH} \\ \\ \text{HC}-\text{OH} \\ \\ \text{CH}_2\text{OH} \\ \text{D-Glucosamine} \end{array} $

Monosaccharide components of serum glycoproteins. Quantitatively, galactose, mannose, glucosamine and neuraminic acid are the most important. Neuraminic occurs in human serum as the sialic acid, N-acetyl neuraminic acid.

TABLE 2

AVERAGE PROTEIN BOUND CARBOHYDRATE LEVELS IN SERUM OF NORMAL INDIVIDUALS

	Mg./100 ml.	Grams/100 grams protein
Hexose	114 \pm 3	1.61
Hexosamine	71 \pm 4	1.01
Sialic acid	62 \pm 2	0.84
Fucose	8.9 \pm 0.6*	—
Uronic acid	0.226 \pm 0.075†	—
Total	256	3.46

* Winzler.²⁴

† Kerby.²⁵

Miller *et al.*⁶ studied the uptake of radioactive lysine in eviscerated rats and found no activity in the albumin, but relatively high activity in the γ -globulin and lesser amounts of activity in the α -globulin and β -globulin fractions. This would indicate the synthesis of some, but not all, serum glycoproteins by the liver.

Studies of serum glycoprotein synthesis utilizing radioactive glucose as a tracer have been made by Boström and his co-workers in rabbits⁷ and Spiro in rats.⁸ Data presented by the latter indicate that most of the protein bound glucosamine of serum is synthesized in the liver. A rapid turnover of serum glycoprotein is indicated by both studies.

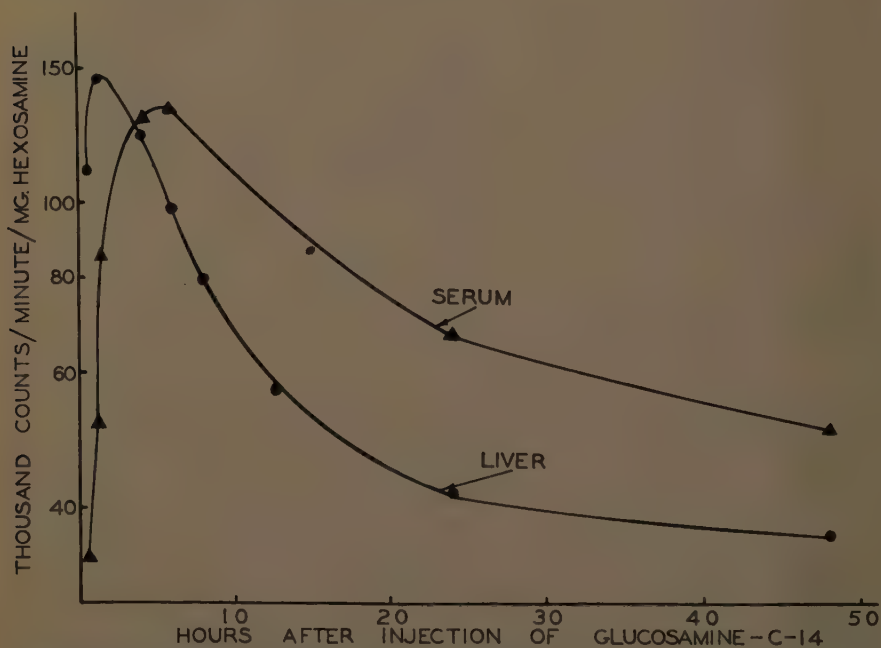


FIGURE 1. Changes of radioactivity in serum proteins and in liver protein after the administration of a single dose of glucosamine-1-C-14 (20 μ c).

Preliminary studies utilizing radioactive glucosamine (glucosamine-1-C-14)* have recently been made in this laboratory (M. R. Shetlar and D. Hearn, unpublished data). The radioactive glucosamine injected intraperitoneally into rats was rapidly incorporated into liver protein-bound glucosamine (FIGURE 1) and into various serum fractions (TABLE 3). This would appear to be a convenient method for biosynthesis of labeled glycoproteins for use in further research. The activity curve of bound glucosamine reached a peak more quickly in the liver than it did in the serum (FIGURE 1) indicating the synthesis of serum glycoproteins by the liver. Lung, kidney, and heart had patterns of radioactivity in which the maximum activity occurred later than that of serum. In

* Provided through the courtesy of Domenic Iezzoni, Chas. Pfizer & Co., Brooklyn, N. Y.

agreement with the studies of Spiro,⁸ a rapid turnover was indicated by the changes of radioactivity of the serum with time. Further work will be required to establish whether the various fractions labeled with radioactive glucosamine have different half lives than those found by labeling the corresponding fractions with I-131, C-14, or S-35. By this method of labeling, it is of interest that the neutral hexoses (mannose and galactose) had little radioactivity (TABLE 4). Data presented in TABLE 4 were further confirmed by paper chromatography studies.

TABLE 3
RADIOACTIVITY IN SERUM FRACTIONS AFTER ADMINISTRATION
OF GLUCOSAMINE-1-C-14 TO RAT*

	Protein (cpm/mg.)
Total protein	2,900
Seromucoid	8,900
α_1 -globulin	8,000
α_2 -globulin	5,900
β -globulin	2,800
γ -globulin	1,100
Albumin	400

* Material administered intraperitoneally in divided doses over a 5-day period.

TABLE 4
RADIOACTIVITY IN THE HYDROLYSATES OF THE BOUND CARBOHYDRATES OF SERUM PROTEIN
OF THE RAT AFTER THE ADMINISTRATION OF GLUCOSAMINE-1-C-14*

Rat No.	cpm/mM		Ratio
	Hexosamine	Hexose	
17	348	19	18.1
18	500	12	41.6

Molar Ratio of Hexosamine to Hexose in Serum — 0.62.

* One hundred microcuries given in divided dosages over 5 days. Hexosamine isolated by ion exchange and paper chromatography. Hexose prepared by ion exchange chromatography.

Origin of the Carbohydrate Component of Glycoprotein

Many, if not all, of the carbohydrates found in serum glycoproteins are generally assumed to arise by biosynthetic means from glucose. Glucose has been shown to be a precursor of all of the various monosaccharides found in serum glycoproteins, and pathways are known for such biosynthesis⁹ (FIGURES 2 and 3). It should be noted that none of these transformations involve breaking of the 6-carbon chain of glucose. The diagrams also indicate that dietary mannose and galactose might be utilized directly for incorporation into glycoproteins. Likewise, glucosamine or acetyl glucosamine, whether from dietary sources or from metabolic breakdown of glycoproteins, might be utilized in this process. Recent work in this laboratory (TABLE 5) has indicated that radio-

active acetyl glucosamine fed to rats by stomach tube is incorporated into protein bound hexosamine. Spiro⁸ has indicated that the turnover rate of bound glucosamine of the blood is too rapid to be accounted for by synthesis from glucose suggesting that previously synthesized glucosamine may be

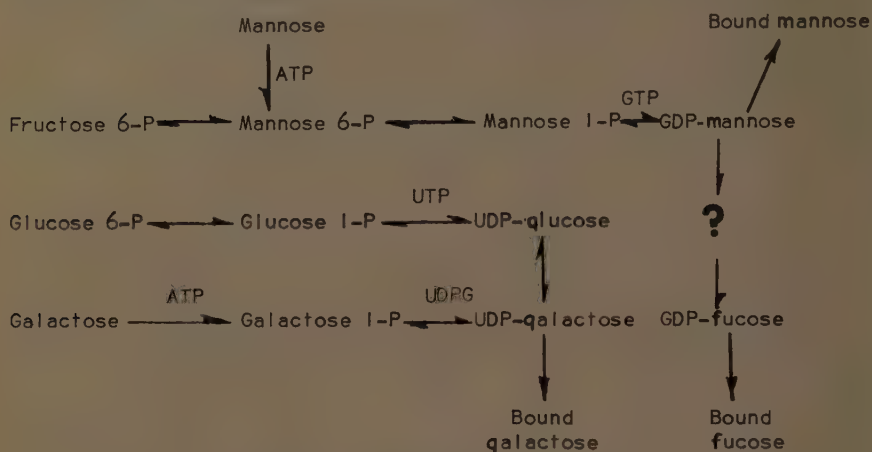


FIGURE 2. Bound hexose biosynthesis.

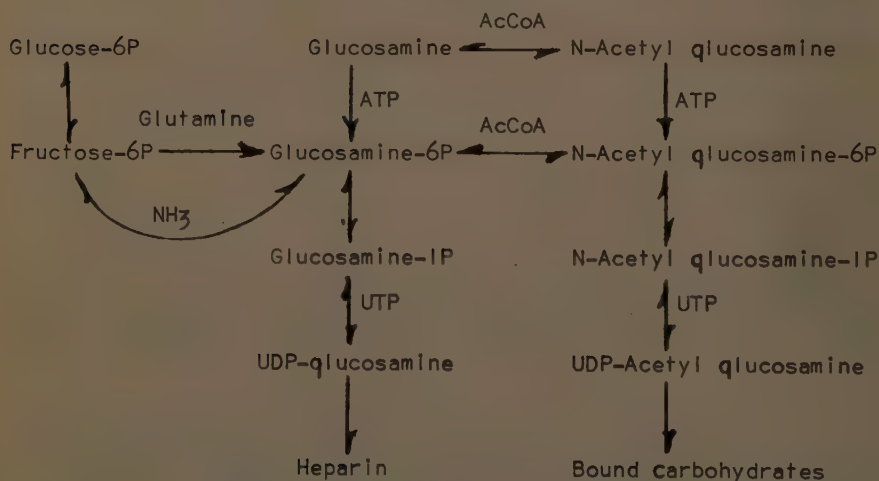


FIGURE 3. Bound hexosamine biosynthesis.

reutilized. The data cited above concerning the incorporation of radioactive glucosamine administered intraperitoneally provides direct evidence that such a mechanism exists. Little is known about the mechanism by which the carbohydrate moieties are incorporated into the protein molecule. By analogy to polysaccharide synthesis, it may probably be safely assumed that sugar nucleotides serve as intermediates. At one time it was assumed by many investigators that the carbohydrate probably existed as a polysaccharide.

However, it is now known that some glycoproteins contain only enough carbohydrate for the existence of oligosaccharides. Ovalbumin is a case in point where the carbohydrate moiety appears to be an oligosaccharide of 6 monosaccharide residues, a tetramannose diglucosamine. The work of Rosevear and Smith¹⁰ indicates that γ -globulin has only 1 carbohydrate residue, a large oligosaccharide or a small polysaccharide of 5 or 6 mannose, 3 galactose, 6 glucosamine, 2 fucose, and 1 sialic acid residues. Considering the average molecular weight of γ -globulin and its hexose content of 1.2 per cent, only one of these residues would be present in each mole of γ -globulin. However, it is perhaps misleading to consider that these average analytical figures apply to all γ -globulins as there is ample evidence that different γ -globulin fractions have different hexose contents.

It has been suggested that orosomucoid, which contains approximately 24 galactose, 12 mannose, 24 acetyl hexosamine, 16 sialic acid, and 2-4 fucose residues in each molecule carries its carbohydrate in small oligosaccharide units,¹¹ with at least 16 units per molecule of protein. Evidence for this view

TABLE 5

ABSORPTION AND UTILIZATION OF ACETYL GLUCOSAMINE-1-C-14 AFTER FEEDING TO RAT*

	Total cpm	Per cent of total administered
Stomach	358×10^3	2.7
Small intestine	116×10^3	0.9
Cecum	162×10^3	1.2
Large intestine	28×10^3	0.2
Serum protein	700×10^3	5.3
Liver	307×10^3	2.3

* Samples taken 8 hours after administration of material by stomach tube.

is that all of the 16 sialic acid residues are removed by neuraminidase and are, therefore, terminal on the carbohydrate chain.

The type of chemical bonds linking the carbohydrate portion to the protein is of great interest. In contrast to the acid mucopolysaccharide-protein complexes where the polysaccharide may be separated from the protein by relatively mild treatment with alkali, the carbohydrate bound to serum protein is broken only by hydrolytic procedures with acid, alkali, or enzymes that also split peptide bonds. This would appear to rule out the occurrence of ester bonds in serum glycoproteins. Masamune¹² has cited evidence of the existence of two carbohydrate-protein bonds, one an ether linkage resulting from the reaction of a hydroxyl group of serine and a hydroxyl of a monosaccharide residue, while the other, a *N*-glycosidic bond, results from the reaction between the hemiacetal hydroxyl of acetyl glycosamine and the free amino group of a terminal aspartic acid. Werner¹³ has suggested an O-glucosidic bond between the reducing group of a monosaccharide residue and the hydroxyl of a hydroxyl amino acid. Rosevear and Smith,¹⁰ working with a peptide obtained by papain hydrolysis of γ -globulin, present data indicating that the carbohydrate is bound by an amide (therefore, presumably involving hexosamine) to the β -carboxyl group of an aspartic acid residue. They also suggest an ester linkage.

Causes of Elevations in Physiological and Pathological Conditions

The serum glycoprotein level is quite constant in an individual under normal conditions (TABLE 6), but is changed slightly by even minor ailments. In

TABLE 6
VARIATION OF SERUM GLYCOPROTEIN IN ONE INDIVIDUAL OVER A 10-YEAR PERIOD

	Serum glycoprotein hexose		Serum protein (gm./100 ml.)	Seromucoid hexose (mg./100 ml.)
	Mg./100 ml.	Hexose: pro- tein ratio*		
Average normal (48 samples)	116	1.66	7.02	16
Range	103-128	1.40-1.77	6.23-7.77	12-18
<i>Respiratory infection</i>				
with fever (1)	162	2.15	7.52	23
(2)	164	2.11	7.78	21
<i>Respiratory infection</i>				
without fever	139	1.77	7.89	—

* Grams of bound hexose per 100 gm. of serum protein.

TABLE 7
CHANGES OF SERUM GLYCOPROTEINS IN DISEASED STATES*

Condition	No.	Serum glycoprotein hexose		Seromucoid hexose (mg./100 ml.)
		Mg./100 ml.	Hexose: pro- tein ratio†	
Normal	80	114 ± 3	1.59 ± 0.03	11 ± 1
<i>Malignancy</i>	105	171 ± 3	2.69 ± 0.05	28 ± 2
Benign lesions	31	123 ± 3	1.96 ± 0.04	—
<i>Rheumatoid Arthritis</i>				
Inactive	8	150 ± 4	1.9 ± 0.03	20 ± 2
Activity 2	13	176 ± 15	2.26 ± 0.06	30 ± 2
Activity 3 & 4	12	197 ± 6	2.73 ± 0.06	30 ± 1
Psychogenic rheumatism	3	118	1.71	12
Degenerative joint D.	28	134 ± 3	1.85 ± 0.02	13 ± 1
Gout, active	2	188	2.65	22
Gout, inactive	6	138	2.04	15
<i>Rheumatic Fever</i>				
Active	18	205 ± 7	2.78 ± 0.10	30 ± 2
Inactive	21	104 ± 4	1.91 ± 0.06	15 ± 1
<i>Tuberculosis</i>				
Minimal active	6	144 ± 5	2.04 ± 0.07	19 ± 4
Moderate advanced, active	13	161 ± 7	2.21 ± 0.08	20 ± 2
Far advanced, active	23	185 ± 7	2.44 ± 0.08	19 ± 1

* Figures following ± sign are standard errors.

† Grams of bound hexose per 100 gm. of serum protein.

certain pathological conditions (TABLE 7) these serum glycoproteins become strikingly altered. Indeed, it is the establishment of these facts that has led to the current lively interest in this field. The area as it relates to clinical chemistry is now too large to adequately survey here. Excellent reviews in this area by Sary¹⁴ and others have recently appeared. Cancer, rheumatoid arthritis, rheumatic fever, and tuberculosis are diseases characterized by ele-

vated serum glycoprotein and seromucoid levels. Variations of serum glycoprotein in normal individuals are summarized in TABLE 8. A slight increase of serum glycoprotein with age is indicated.¹⁵ The effects of more pathological changes either clinical or subclinical in older individuals have not been carefully evaluated in these studies. A more striking change with age has been noted in rats by Boas and Peterman.¹⁶ The elevation of serum glycoproteins in pregnancy is of interest in that the seromucoid level is not elevated.¹⁷ Any explanation of the significance of elevated serum glycoprotein levels must account for the various changes found in disease and different physiological states.

TABLE 8
SUMMARY OF SERUM GLYCOPROTEIN IN THE NORMAL HUMAN

Age or condition	No. samples	Serum glycoprotein hexose		Seromucoid hexose (mg./100 ml.)
		Mg./100 ml.	Hexose:protein ratio*	
Fetal	15	80 (62-103)	1.41 (1.05-1.77)	6 (3-9)
3-8 yr.	8	105 (94-118)	1.60 (1.47-1.82)	—
21-49 yr.				
Males	18	110 (93-126)	1.58 (1.26-1.90)	
Females	10	111 (100-125)	1.58 (1.42-1.81)	12 (8-18)
61-85 yr.	15	129 (104-138)	1.79 (1.62-2.06)	
Pregnancy (8-10th month)				
Primigravida	13	152 (120-172)	2.46 (2.26-2.63)	
Multigravida	16	150 (123-191)	2.42 (1.96-2.82)	12 (8-14)
Pregnancy (4th month)	6	122 (110-136)	1.87 (1.70-2.10)	

* Grams of bound hexose per 100 gm. of serum protein.

Origin of the Elevated Serum Glycoprotein

A number of hypotheses concerning the physiological mechanisms that cause elevated levels of serum glycoproteins in disease conditions have been proposed and will be discussed on a comparative bases.

*Glycoproteins are released into the blood stream from injured, inflamed, or otherwise altered tissue.*¹⁸ This suggestion has the merit of simplicity. The fact that serum does not contain appreciable amounts of acid mucopolysaccharides even in inflammatory conditions while tissues contain large amounts of these components has been cited as evidence against this hypothesis. However, if one postulates the existence of a mechanism to remove rapidly the acid mucopolysaccharides from serum this objection may be circumvented. In this regard the work of Smith and Kerby¹⁹ is of interest. After intravenous injection of chondroitin sulfate into rabbits, 5 to 10 per cent was excreted in the urine within 2 to 4 days. Fate of the remainder is unknown, but is apparently removed from the serum by other means. On the positive side, there is much evidence that glycoproteins similar to serum components occur in tissue. Release of these components together with retention or removal of acid mucopolysaccharides may be involved in inflammation.

*Glycoproteins arise as a response of the organism to tissue injury²⁰ or proliferation.*²¹ According to this suggestion, injured tissue components initiate a

mechanism that causes increased production of serum glycoproteins. These proteins may have some physiological value in the organism's defense or in the reparative process. In support of this hypothesis may be cited the fact that many types of injury, either clinical or experimental, produce essentially the same response. Recent work of Spiro⁸ using radioactive glucose and that described above using radioactive glucosamine would indicate that the liver is the major organ involved in normal synthesis of serum glycoprotein. The next step in this direction is to determine if this synthetic process of the liver is altered in inflammatory states.

In contrast to inflammatory states, the serum glycoprotein elevations that occur in pregnancy are of interest in that normal seromucoid levels are present in these subjects. It may be tentatively suggested that the influence of inflammation on glycoprotein synthesis is different from that found when tissue proliferation or growth is involved. Various sites of biosynthesis may also be involved.

Glycoprotein in serum becomes elevated because certain tissues (for example cancer tissue) utilize proteins low in bound carbohydrate. This hypothesis was originally suggested by Weimer and his co-workers²² to explain the elevation of seromucoid in rats bearing Walker 256 tumors. It may be enlarged to include other glycoproteins and other pathological conditions. Such conditions may result in a higher utilization of proteins, such as serum albumin, which are low in bound carbohydrates leaving the carbohydrate rich fractions. This is strengthened by the fact that a decrease of serum albumin commonly occurs in conditions where elevated glycoproteins occur. However, it has not always been established whether this is due to a more rapid utilization or to a decrease in production of albumin. The report of a preferential utilization of α_2 -glycoprotein by Walker 256 cancer cells in tissue culture²³ is of interest in regard to this postulation.

It is not possible at this time to evaluate definitely the above hypotheses concerning the origin of serum glycoproteins. It seems feasible that the major origin of the serum glycoproteins may be the liver, and that the rapid rise after inflammation may be explained by increased liver biosynthesis or release of glycoproteins from the liver. However, considering the multiplicity of the serum glycoproteins, other tissues may supply appreciable amounts of glycoproteins to serum, and, to some degree, all of the above hypotheses may well be correct. These questions, as well as the function and fate of these substances, remains to be elucidated. However, with the use of glycoproteins containing radioactively labeled carbohydrate constituents, answers to these questions should be forthcoming.

Summary

The present state of knowledge of the origin and significance of the serum glycoproteins has been reviewed. Preliminary studies were described indicating the direct incorporation of parenterally administered radioactive glucosamine into serum glycoproteins. Comparative serial studies were made of the radioactivity in the bound hexosamine of the liver and serum. These and similar studies with radioactive glucose indicate that the liver is the principal

site of incorporation of hexosamine into the serum proteins in the normal animal. The hypothesis that elevations of serum glycoproteins in pathological conditions represent a systemic response to injury or tissue proliferation would, therefore, appear to be favored, that is, biosynthesis of various glycoproteins by the liver is increased. Other possibilities, that is, (1) glycoproteins are released into the blood stream from injured, inflamed, or otherwise altered tissue, and (2) certain tissues preferentially utilize plasma proteins low in bound carbohydrates, still merit further investigation.

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FATTY ACID COMPOSITION OF THE SERUM LIPOPROTEINS*

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Introduction

Considering the purpose of this monograph, it is appropriate to explore an aspect of the blood lipoproteins that may be potentially related to states of health and disease. This aspect is the detailed fatty acid composition of the human serum lipids and serum lipoproteins.

Approximately 95 per cent of the blood lipids exist in the form of lipoproteins. These lipoproteins range in particle size from approximately 100 to 10,000 Å, as determined by both ultracentrifugal analysis and electron microscopy.¹ Because of their ratio of lipid to protein, they range in hydrated density from approximately 0.93 to 1.15 gm./cc. in density. These lipoproteins may be broadly grouped into the high density lipoproteins and 2 low density lipoprotein groups: the S_f 0 to 20, and the S_f 20 to 10⁵ groups. Already there is evidence that there exists a quantitative statistical relationship between elevated serum concentrations of these 2 classes of low-density lipoproteins and such disease states as coronary heart disease,^{2,3} diabetes, the xanthomatoses, and states of thyroid abnormalities.⁴

During the past few years the broad chemical composition of the serum lipoproteins has been studied⁵⁻⁷ in considerable detail. Thus for each major serum lipoprotein class, the approximate mass per cent content of such lipids as cholesteryl esters, cholesterol, glycerides, phospholipids, and unesterified fatty acids is known. The S_f 0 to 20 lipoproteins contain the major share of the serum cholesteryl esters, and the S_f 20 to 400 lipoproteins carry a major share of the serum glyceride content. However it should be noted that approximately two thirds of the serum lipids by weight are actually fatty acids, existing either in form of unesterified fatty acids or as fatty acid esters in cholesteryl esters, glycerides, and phospholipids. Thus we may consider approximately 50 fatty acids as potential building blocks of the lipoproteins. Therefore each class of fatty acid ester, cholesteryl ester, glyceride, or phospholipid is a complex mixture of many biochemical compounds, differing in their fatty acid moieties. A fatty acid analysis by gas-liquid chromatography (GLC) of each chemical class isolated from serum or from isolated serum lipoproteins allows study of this biochemical complexity. Certain questions that we hope to begin to answer from these analyses are: (1) What are the characteristic fatty acid compositions in each chemical class of serum?; (2) Are the fatty acids present in each chemical class the same for all lipoprotein classes?; (3) What is the variability of these fatty acid compositions from person to person, as well as in the same individual, over a period of time?; and (4) Are these compositions related to any diseases, particularly to those in which there may be a disturbance in lipid metabolism?

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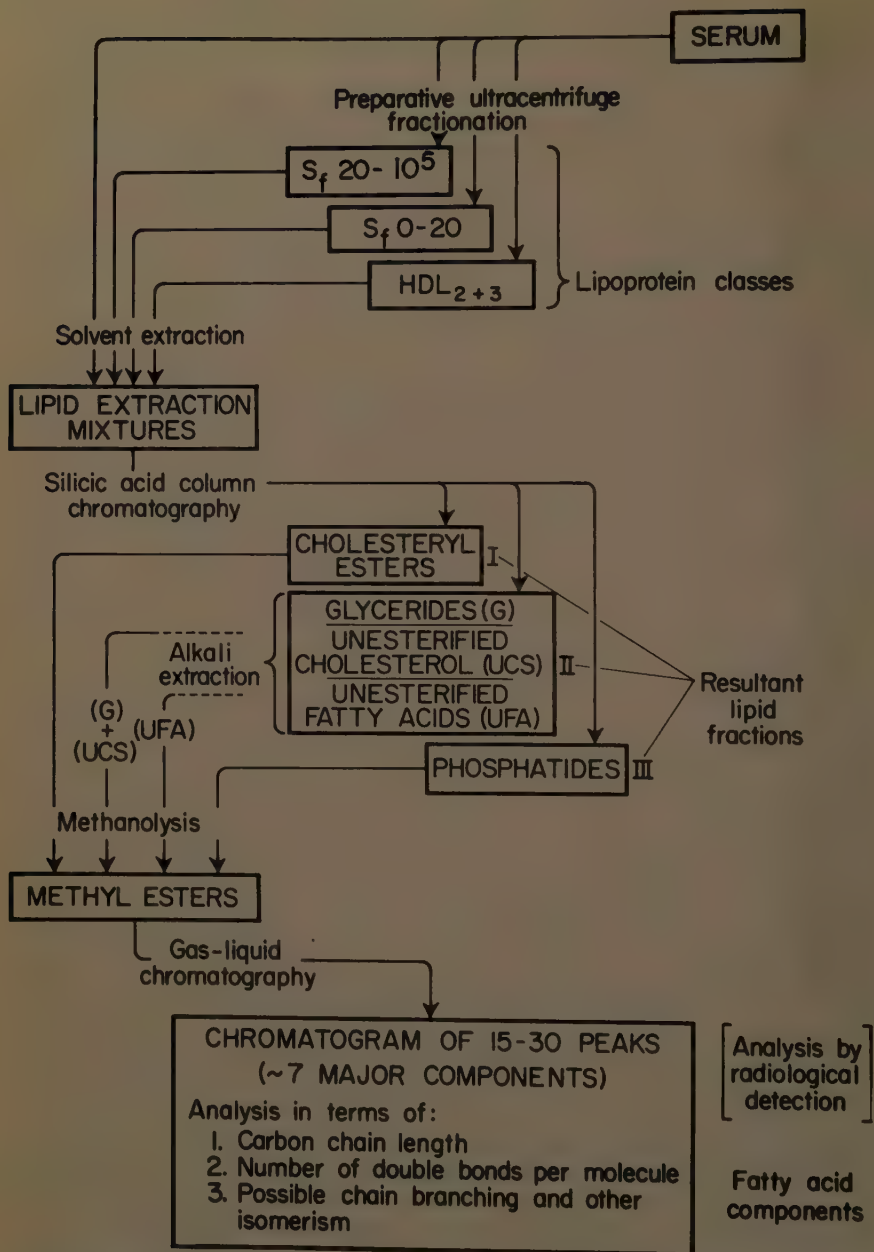


FIGURE 1. Scheme for fatty acid analysis of the serum lipids and lipoproteins. Reprinted by permission of Freeman *et al.*²⁶

Methods

FIGURE 1 illustrates the complete analytical scheme that we have used for fatty acid analysis of the blood lipids. First, in our lipid studies, the serum is directly extracted⁸ and separated by silicic acid chromatography with evaluation of fractions by infrared spectrophotometry.⁹ This procedure¹⁰⁻¹³ with modifications employs 1 per cent diethyl ether in normal hexane, chloroform, and methanol as eluting solvents. The 3 chemical fractions obtained are: (1) cholesteryl esters, (2) a mixture containing glycerides, cholesterol, and unesterified fatty acids, and (3) the phospholipids. These separated lipids are converted to the methyl esters for gas-liquid chromatography by methanolysis.¹⁴ To study the chemical fractions within each lipoprotein group, the serum is first separated by successive preparative ultracentrifugation¹⁵ into the 3 major lipoprotein fractions: the glyceride-rich S_f 20 to 10⁵, the cholesterol-rich

TABLE 1
REPRODUCIBILITY OF TEN CONSECUTIVE SAMPLE INJECTIONS,* USING THE
BECKMAN LIQUID SAMPLER

Injection	Elution time (min.)	Triangulated area at 1 v (in. ²)	Peak height at 1 v (in.)
1	8.80	0.66	1.88
2	8.83	0.67	1.94
3	8.80	0.66	1.91
4	8.83	0.66	1.94
5	8.83	0.68	2.03
6	8.84	0.65	1.84
7	8.78	0.66	1.92
8	8.87	0.64	1.92
9	8.81	0.67	1.89
10	8.84	0.66	1.93
Mean	8.82	0.66	1.92

* Consisting of 0.01 ml. *N* hexane containing 10.3 μ g. methyl palmitate.

Reprinted by permission of Upham *et al.*²⁵

S_f 0 to 20 low-density lipoproteins, and the phospholipid-rich, high-density lipoprotein group. The remaining analytical procedure is identical to that of the serum fatty acid analysis. Where studied, the unesterified fatty acids are obtained by alkali extraction¹¹ from the second silicic acid fraction.

The fatty acid methyl ester samples were analyzed by gas-liquid chromatography. Our GLC apparatus and detector, patterned after that described by Lovelock,¹⁶ is described in detail by Upham *et al.*¹⁷ We inject our methyl ester samples with a modified Beckman liquid sampler in 5 to 20 μ l. *N* hexane.

TABLE 1 illustrates injection reproducibility of 10 μ g. of methyl palmitate in 10 μ l. of hexane. The retention time varies less than 1 per cent, the area triangulated by less than 3 per cent, and the peak height by less than 6 per cent. FIGURE 2 illustrates a typical calibration curve for methyl palmitate obtained with our β -particle detector over the mass range of 0.02 to 42 μ g. For this calibration, methyl palmitate dissolved in normal hexane was diluted successively by a factor of 2 (each time). All injections over this mass range were of equal volume (0.01 ml.), insuring maximum accuracy in the mass of

injected methyl ester. The relationship observed between peak height and sample mass is not linear over this range but is somewhat sigmoidal. This relationship is also true if one uses triangulated area instead of peak height. Furthermore, it appears that the calibration characteristics, including the

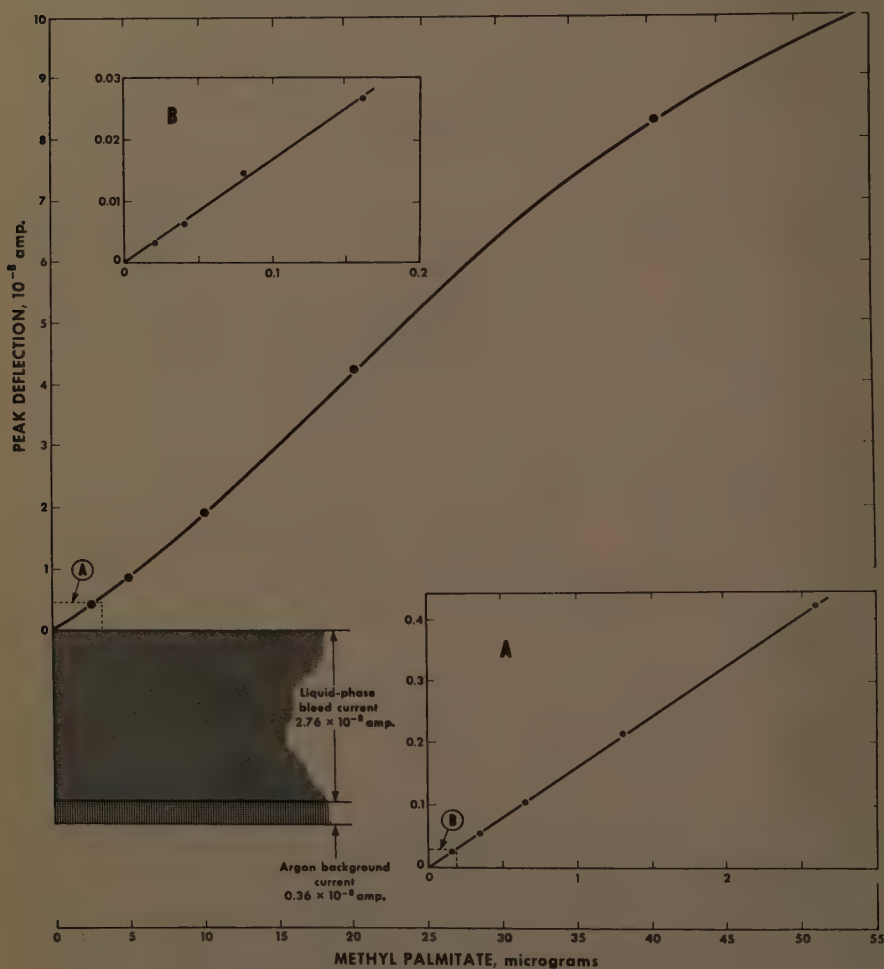


FIGURE 2. Calibration curve for methyl palmitate.

degree of sigmoidal distortion of the methyl ester calibration curve, depend on the amount and composition of the liquid phase bleed. We operate our detector as a nonlinear instrument making appropriate calibration corrections.¹⁸ Above 1×10^{-7} amp. (above full-scale deflection), our detector saturates with a minimal increase in peak deflection with increase in sample mass (FIGURE 2). During the conditions of the above calibration our detector collects approximately 1 electron for each 4000 mol. of methyl palmitate.

FIGURE 3 shows a typical chromatogram obtained from a serum phospholipid sample. Because the peaks broaden with time following injection, additional sensitivity is required to resolve adequately the later chromatographic components. Recorder sensitivity has been switched to $10\times$ following methyl linoleate and to $33.3\times$ shortly after methyl arachidonate. Since the number of resolvable components ordinarily present on each chromatogram ranges from 15 to 30, there is clearly a formidable problem of tabulating, presenting, and comparing data. However, 80 to 90 per cent by mass of the component fatty acid present on each chromatogram may be identified with 6 known methyl ester elution times, namely, methyl palmitate, palmitoleate, stearate,

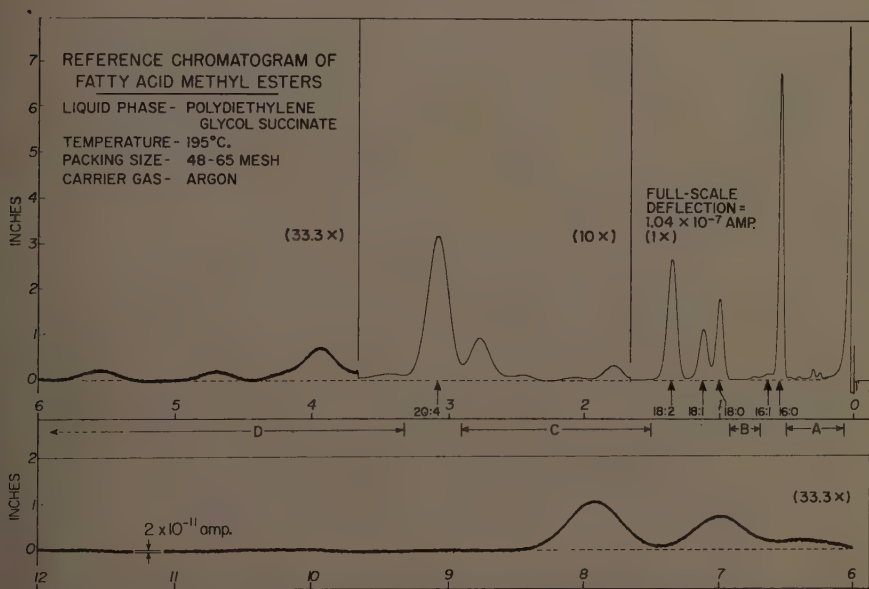


FIGURE 3. Typical chromatogram of fatty acid methyl esters from a serum phospholipid fraction. The retention time for methyl stearate is 12.07 min. Reprinted by permission of Nichols *et al.*²⁷

oleate, linoleate, and arachidonate. To allow presentation and comparison of data, the remaining 10 to 20 per cent of methyl esters have been grouped tentatively into the 4 classification bands: prepalmitate (A), palmitoleate to stearate (B), linoleate to arachidonate (C), and postarachidonate (D).

We employ a technique¹⁸ for analysis of chromatograms using punched cards together with an appropriate computer (IBM 650). In essence each chromatogram, consisting of a sequence of peaks, is reduced to a small deck of IBM-punched cards: one for each chromatographic component. The basic data placed on these input IBM-punched cards consists of the elution time, the peak height, and amplification of each chromatographic component. Because the triangulated peak width is roughly proportional to the elution time, it is possible to calculate the approximate area of each component by the product of the elution time and the peak height. However, since the re-

lationship between peak height and component mass is sigmoidal rather than linear, a correction function is needed. This first correction function, obtained on columns using polydiethylene glycol succinate (PDEGS) as a liquid phase for our operating conditions, is shown in FIGURE 4. This function primarily corrects for nonlinearity of all calibration curves, irrespective of the differences in detector response for different fatty acid methyl ester components.

CORRECTION FACTOR

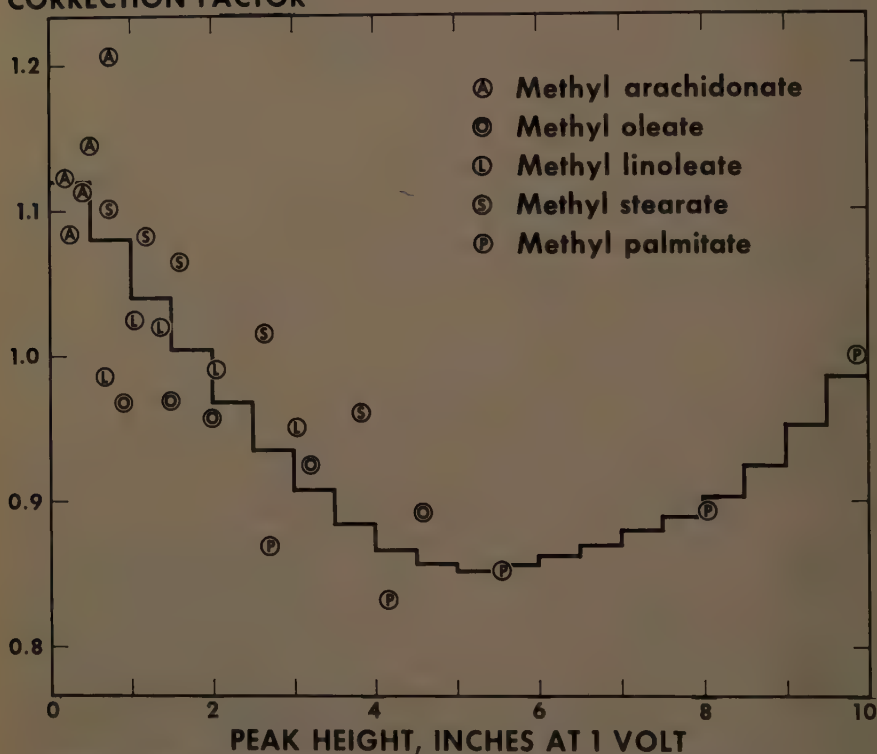


FIGURE 4. First correction function for computer analysis of chromatograms. Reprinted by permission of Tandy *et al.*²⁸

A further refinement is desirable, and it is provided by a second correction function (FIGURE 5). This function corrects on the basis of relative retention time and is obtained from calibration data of methyl ester components of from methyl caprate (10:0) to methyl arachidonate (20:4).

TABLE 2 shows the results obtained by this procedure, using a standard calibration mixture over the range of 1 to 230 μg . sample size. Although quantitation of trace components as well as inadequately separated components still presents difficulties, all principal methyl ester components are quantitated to within approximately ± 5 per cent.

When using the PDEGS liquid phase at 195° C., the presence of unesterified cholesterol in the methyl ester samples from both the cholesteryl ester

CORRECTION FACTOR

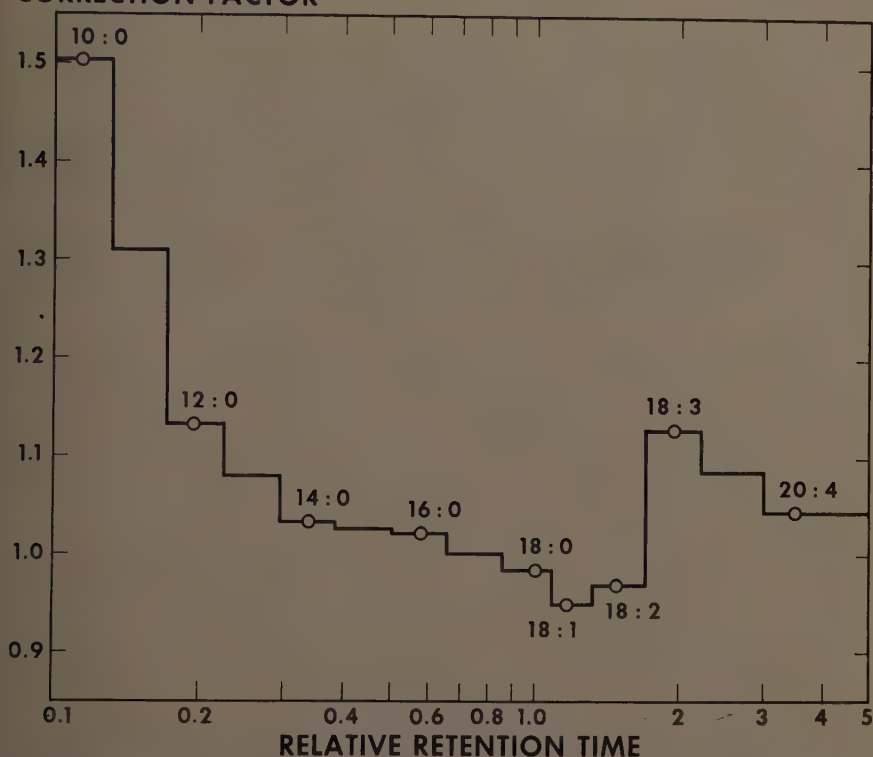


FIGURE 5. Second correction function for computer analysis of chromatograms. Reprinted by permission of Tandy *et al.*²⁸

TABLE 2
CALIBRATION WITH STANDARD METHYL ESTER MIXTURE*

Component	1	2	3	4	5	6	7	8	9	Mean values	Actual mass (%)
10:0	1.9	1.0	1.0	1.0	1.0	0.9	0.9	0.9	0.8	1.0	1.0
12:0	1.3	1.7	1.6	1.3	1.7	1.7	1.7	1.7	1.6	1.6	1.9
14:0	1.9	2.0	2.0	1.9	2.0	2.0	2.0	2.0	1.9	2.0	2.5
16:0	22.6	24.0	23.4	23.6	22.8	23.1	23.1	23.8	25.8	23.6	23.0
18:0	15.7	15.2	15.9	16.1	15.6	15.8	15.9	15.7	15.5	15.7	15.6
18:1	18.2	19.6	19.3	19.9	19.0	19.3	19.5	19.5	18.8	19.2	19.2
18:2	17.0	16.9	17.4	17.3	16.4	16.4	16.4	16.1	16.3	16.7	16.1
18:3	3.1	4.7	4.6	4.7	4.4	4.4	4.6	4.3	4.1	4.3	4.4
20:4	18.2	14.9	14.8	14.4	14.4	14.3	14.1	14.3	13.6	14.8	14.1
Minor components	—	—	—	—	2.6	2.0	1.8	1.8	1.6	1.1	2.3
Total mass analyzed (μg.)	0.93	1.74	3.58	6.95	14.5	28.5	56.9	116.0	232.3	—	—

* Mass per cent determination.

and glyceride-containing lipid fractions definitely interferes¹⁵ in the gas chromatographic analysis. When present, cholesterol interferes beyond stearate times of approximately 6, as well as preventing any subsequent analysis before 12 stearate times have passed. However, no significant interference is encountered before 6 stearate times. With the exception of Case 6 (see lipoprotein results), our data are for methyl ester samples without unesterified cholesterol removal and therefore represent adequate analysis of these fractions only up to 6 stearate times. Both silicic acid chromatography and microsublimation¹⁴ allow separation of the methyl esters from unesterified cholesterol. However, each of these procedures may introduce both losses and fractionating effects unless rigorous control measures are maintained.

TABLE 3
FATTY ACID COMPOSITION OF THE SERUM CHOLESTERYL ESTER FRACTION

Sample	1	2	3	4	5	6	7	Mean
Age, sex.....	52, F	51, M	38, M	41, M	34, M	40, M	41, M	
Pre 16:0 (A)	5.3 ^{11*}	2.8 ¹¹	3.5 ¹¹	2.2 ¹¹	2.3 ¹³	1.5 ¹⁰	6.3 ¹¹	3.4 ¹¹
16:0	13.7	10.7	8.9	10.3	8.8	9.0	11.0	10.4
16:1	7.7	2.5	2.4	3.1	1.7	2.8	3.1	3.3
16:1-18:0 (B)	2.0 ²	0.7 ²	1.3 ²	0.7 ²	0.8 ²	0.6 ²	1.8 ²	1.1 ²
18:0	1.6	1.1	1.2	1.1	1.0	0.8	1.9	1.2
18:1	23.3	17.5	15.0	17.2	12.9	17.5	16.7	17.2
18:2	34.4	57.4	59.2	52.2	64.1	61.5	52.5	54.5
18:2-20:4 (C)	3.8 ⁴	1.4 ³	2.7 ³	3.4 ³	1.7 ³	1.5 ³	2.2 ⁵	2.4 ³
20:4	7.3	5.6	5.1	8.8	6.3	4.1	4.6	6.0
Post 20:4 (D)	1.0 ²	0.4 ¹	0.7 ²	1.0 ²	0.6 ³	0.6 ²	—	0.6 ²
Total components analyzed	25	23	24	24	27	23	24	24

* Superscripts indicate total components resolved within each classification band.

Results: Serum Fractions

The results for the lipid fractions obtained from 7 serum samples are given in TABLES 3 to 5. All values are given as mass per cent of total methyl esters determined from each gas chromatogram. The results show a definite pattern of fatty acid composition for each chemical fraction obtained from all 7 subjects. Some variations in fatty acid composition for the same chemical fraction are observed from person to person. This might be anticipated from the studies of Ahrens *et al.*,¹⁹ in which differences in fatty acid distributions were observed as a result of changes in the composition of the dietary fat. However, a definite pattern of composition is observed among all subjects for each chemical class. For the cholesteryl ester fractions, the predominant fatty acids are linoleic (55 per cent), oleic (17 per cent), palmitic (10 per cent), and arachidonic (6 per cent) acid. Thus cholesterol esters are an important biochemical form for the transport of the more highly unsaturated fatty acids. In contrast, serum glycerides contain a considerably higher content of the more saturated fatty acids. The principal fatty acids present in the glyceride-containing fractions are oleic (38 per cent), palmitic (31 per cent), and linoleic

(16 per cent) acid. The phospholipid fractions show another distinct fatty acid pattern. The major fatty acids are palmitic (34 per cent), linoleic (21 per cent), stearic (14 per cent), oleic (11 per cent), and arachidonic (10 per cent) acid. Compared to the other chemical fractions of serum, phospholipids are associated with an unusually high content of both palmitic and stearic acid. Finally, the unesterified fatty acid fraction, primarily transported in the serum bound with serum albumin, shows still another fatty acid distribu-

TABLE 4
FATTY ACID COMPOSITION OF SERUM GLYCERIDE-CONTAINING FRACTION

Sample	1	2	3	4	5	6	7	Mean
Pre 16:0 (A)	4.1 ⁸	3.8 ⁷	4.5 ⁶	4.8 ⁸	2.9 ⁸	2.0 ⁷	4.1 ⁸	3.7 ⁷
16:0	36.4	28.2	29.9	33.1	27.2	34.4	27.5	30.9
16:1	5.5	3.9	3.4	4.2	3.4	3.3	3.4	3.9
16:1-18:0 (B)	1.3 ²	1.3 ²	1.1 ²	1.3 ²	1.0 ²	0.7 ²	0.9 ²	1.1 ²
18:0	4.5	5.2	4.1	5.3	5.0	3.3	5.1	4.6
18:1	37.2	38.2	36.0	35.6	36.7	39.6	40.5	37.7
18:2	9.1	17.2	18.9	12.8	20.7	14.5	15.5	15.5
18:2-20:4 (C)	1.2 ¹	1.1 ¹	0.9 ¹	1.5 ²	1.5 ²	1.3 ²	1.7 ²	1.3 ²
20:4	0.8	1.3	1.1	1.6	1.8	0.9	1.4	1.3
Post 20:4 (D)	—	—	—	—	—	—	—	—
Total components analyzed	17	16	15	18	18	17	18	17

TABLE 5
FATTY ACID COMPOSITION OF THE SERUM PHOSPHOLIPID FRACTION

Sample	1	2	3	4	5	6	7	Mean
Pre 16:0 (A)	2.0 ⁹	1.8 ⁸	1.5 ⁸	2.7 ¹¹	2.6 ⁹	1.7 ⁹	2.7 ⁹	2.1 ⁸
16:0	30.9	34.2	39.0	34.7	34.4	32.0	32.4	33.9
16:1	1.5	0.9	0.9	1.2	1.1	1.0	1.2	1.1
16:1-18:0 (B)	0.9 ²	0.9 ²	0.8 ²	0.9 ²	1.1 ²	0.8 ²	1.1 ²	0.9 ²
18:0	15.1	15.1	12.6	13.9	14.8	12.5	14.5	14.1
18:1	16.1	9.8	10.0	9.2	9.3	11.6	12.8	11.3
18:2	20.0	23.5	23.0	17.8	21.1	21.7	21.4	21.2
18:2-20:4 (C)	3.8 ²	2.9 ²	3.1 ³	4.8 ³	4.1 ³	3.2 ⁴	3.6 ⁴	3.6 ³
20:4	9.8	9.3	8.0	13.3	11.0	7.8	8.8	9.7
Post 20:4 (D)	—	1.7 ²	1.2 ¹	1.5 ²	0.5 ¹	7.7 ⁶	1.4 ³	2.0 ³
Total components analyzed	19	17	18	24	21	27	24	21

tion. TABLE 6 shows (4 cases) that the major fatty acids for this fraction are oleic (25 per cent), palmitic (26 per cent), and linoleic (15 per cent) acid. Also the short chain fatty acids (prepalmitate group) appear to be higher (9 per cent) than in any other fatty acid-containing fraction.

TABLES 6 and 7 allow comparison, where possible, of our data with those of James *et al.*,²⁰ Dole *et al.*,²¹ and Hallgren *et al.*²² However, only in the latter study were cholesterol esters and glycerides separated. In general there is agreement in the profile of fatty acid composition obtained, although some discrepancies do exist. Apart from the anticipated variability between the in-

dividuals studied, some of these differences may be the result of different procedures used for sample preparation as well as gas-liquid chromatographic calibration difficulties.

TABLE 6
FATTY ACID COMPOSITION OF THE SERUM UNESTERIFIED FATTY ACID FRACTION

Sample	1	2	3	4	Mean (4 cases)	Dole <i>et al.</i> ²¹ (18 cases)
Age, sex.....	71, M	25, M	52, F	41, M		
Pre 16:0 (A)	7.4 ⁸	7.6 ⁸	10.0 ⁸	9.1 ⁴	8.5 ⁷	2.6
16:0	24.5	29.3	21.9	26.0	25.5	25.6
16:1	4.8	4.1	3.5	5.1	4.4	—
16:1-18:0 (B)	1.6 ²	1.8 ²	2.0 ²	3.4 ²	2.2 ²	—
18:0	9.5	9.8	9.6	10.4	9.8	12.9
18:1	28.1	24.9	23.0	22.4	24.6	28.9
18:2	12.5	14.9	20.7	13.3	15.3	14.5
18:2-20:4 (C)	4.5 ⁴	2.6 ⁵	5.8 ³	5.1 ³	4.5 ⁴	—
20:4	2.9	3.0	0.6	1.7	2.1	3.4
Post 20:4 (D)	4.2 ²	2.1 ³	2.8 ²	3.5 ²	3.2 ²	—
Total components analyzed	22	24	21	17	21	—

TABLE 7
COMPARISON OF FATTY ACID COMPOSITION OF SERUM FRACTIONS

Component	Our data (7 cases)			James <i>et al.</i> ²⁰ (12 cases)	Dole <i>et al.</i> ²¹ (6 cases)	Hallgren <i>et al.</i> ²² (5+ cases)		
	Choles- teryl esters	Glycer- ides	Phospho- lipids	Phospho- lipids	Phospho- lipids	Phospho- lipids	Choles- teryl esters	Glycer- ides
Pre 16:0 (A)	3.4 ¹¹	3.7 ⁷	2.1 ⁸	1.1	2.1	1.1	2.0	3.9
16:0	10.4	30.9	33.9	26.7	36.6	27.7	11.0	24.9
16:1	3.3	3.9	1.1	2.0	2.5	—	4.8	6.2
16:1-18:0 (B)	1.1 ²	1.1 ²	0.9 ²	—	—	1.6	1.1	1.8
18:0	1.2	4.6	14.1	13.3	12.8	11.9	0.8	4.0
18:1	17.2	37.7	11.3	20.8	18.6	14.3	23.2	41.4
18:2	54.5	15.5	21.2	18.8	16.0	20.4	46.2	10.9
18:2-20:4 (C)	2.4 ³	1.3 ²	3.6 ³	—	—	2.4	2.1	1.4
20:4	6.0	1.3	9.7	9.7	4.0	8.8	5.9	0.8
Post 20:4 (D)	0.6 ²	—	2.0 ³	—	—	7.8	2.4	2.3
Total components ana- lyzed	24	17	21	—	—	—	—	—

Results: Fatty Acids in Lipids of the Major Lipoprotein Classes

The results of this preliminary study were obtained from serum fractionated from 7 adult subjects. Serum samples 1 and 2 were obtained, 1 month apart, from the same individual. It is noteworthy that over such a comparatively short interval these samples show marked differences in fatty acid composition. Samples 7 and 8 are from patients with xanthoma tuberosum and xanthoma tendinosum respectively.

TABLES 8A, B, and C show the fatty acid distribution obtained from the cholesteryl ester moiety of the three principal lipoprotein classes. Comparison of the fatty acid distributions among the different lipoproteins for each case show close similarity between the S_f 0 to 20 and HDL_{2+3} class lipoproteins. For the 8 samples studied, there appears to be no consistent pattern for the very low density S_f 20 to 10^5 lipoprotein group. However, specific differences (when present) may be characterized by an elevated level of palmitic, stearic, and oleic acid, as well as a reduced level of linoleic and arachidonic acid relative to the two higher density lipoprotein classes. When the 8 samples are compared with each other the data show general similarity in fatty acid distribution for both the S_f 0 to 20 and HDL_{2+3} groups.

TABLES 9A, B, and C show the fatty acid distribution observed for the glyceride-containing lipid fraction obtained from each lipoprotein group. From person to person, these fractions showed comparatively minor differences in fatty acid distribution. If we consider each sample, we observe very close similarity between the S_f 0 to 20 and the HDL_{2+3} lipoprotein classes. In the glyceride-containing fraction isolated from each lipoprotein group, the levels of the prepalmitic acid group are higher than either of the other two chemical fractions studied. However, there are uncertainties in the measurement of the short-chain fatty acids, since they may be selectively lost during the processing of the lipid samples because of their higher volatility. Also the use of a solvent to inject the methyl ester samples introduces base-line uncertainties in the very early portion of the chromatogram and usually does not allow reliable quantitation of components of shorter chain length than 10:0.

TABLES 10A, B, and C show the fatty acid distribution present in the phospholipid fractions obtained from each lipoprotein class. With the exception of sample 2, the fatty acid distribution of the phospholipid moiety of the 3 ultracentrifugal fractions of each sera is broadly similar. The S_f 20 to 10^5 lipoproteins of sample 2 show a relatively high palmitic and stearic acid content as well as a lower linoleic and arachidonic acid content. However, sample 2 shows a similar fatty acid composition for the S_f 0 to 20 and HDL_{2+3} lipoprotein fractions. Comparison of each case with the other does show over-all variability in fatty acid composition. Case 4 for instance shows a higher content of palmitic acid and an unusually low content of arachidonic acid for each of the 3 lipoprotein fractions. Also these preliminary data suggest that the highest level of the postarachidonic acid group may be found in the phospholipid moiety of the S_f 0 to 20 class lipoproteins. This could be the result of the relatively high content of sphingomyelins in the S_f 0 to 20 lipoprotein class. Preliminary work²³ has shown isolated sphingomyelins to contain a high percentage of these fatty acids with elution times longer than methyl arachidonate. However, it should be noted that the methanolysis procedure¹⁴ employed in these studies accomplishes only partial transesterification of sphingomyelins.

Discussion

It is noteworthy that Cases 7 and 8 (of the lipoprotein series) are patients with xanthoma tuberosum and xanthoma tendinosum respectively. These

TABLE 8A
FATTY ACID COMPOSITION OF THE CHOLESTERYL ESTER FRACTION S_I
20 to 10⁵ LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Age, sex.....	38, M	38, M	21, F	44, M	49, M	55, M	43, M	73, F	
Pre 16:0 (A)	2.7 ⁹	3.7 ⁹	6.2 ⁹	1.8 ⁵	1.8 ²	2.7	0.5 ³	2.3 ⁵	2.7 ⁶
16:0	13.9	29.2	24.4	26.6	12.4	10.8	11.7	28.4	19.7
16:1	4.5	2.0	2.3	3.0	4.2	3.3	2.4	4.4	3.3
16:1-18:0 (B)	0.9 ¹	1.2 ²	2.0 ²	0.9 ²	1.5 ²	1.4 ²	0.5 ²	0.5 ¹	1.1 ²
18:0	2.3	6.8	10.8	4.2	2.6	3.1	2.3	6.6	4.8
18:1	25.5	35.4	26.8	44.9	23.3	25.3	18.9	36.6	29.6
18:2	44.6	18.2	23.4	15.1	41.9	40.4	52.1	16.4	31.5
18:2-20:4 (C)	2.5 ²	1.9 ⁵	2.2 ⁴	2.2 ⁵	4.8 ⁴	3.3 ⁵	3.1 ³	2.4 ⁴	2.8 ⁴
20:4	3.1	1.5	2.1	1.4	7.0	6.5	7.9	2.0	3.9
Post 20:4 (D)	—	0.3 ¹	—	—	0.6	3.2 ⁴	0.7 ²	0.5 ²	0.7 ¹
Total components analyzed	18	23	21	18	15	24	16	18	19

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 8B
FATTY ACID COMPOSITION OF THE CHOLESTERYL ESTER FRACTION S_I 0 TO 20 LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Pre 16:0 (A)	7.2 ¹⁰	1.7 ⁹	2.4 ⁶	1.2 ⁶	1.1 ²	1.5 ⁸	0.7 ⁴	1.3 ⁵	2.1 ⁶
16:0	11.1	11.2	11.2	11.4	12.7	11.4	11.1	13.2	11.7
16:1	4.2	2.8	3.2	3.3	4.2	3.6	2.4	5.0	3.6
16:1-18:0 (B)	1.3 ¹	0.7 ²	1.2 ²	0.7 ²	0.7 ²	0.7 ²	0.4 ²	0.3 ¹	0.7 ²
18:0	1.7	1.2	1.2	1.3	1.4	1.1	1.4	1.4	1.4
18:1	16.7	19.1	18.9	19.9	19.7	21.6	15.5	21.4	19.1
18:2	48.7	54.1	52.3	49.5	46.6	47.1	56.3	44.0	49.8
18:2-20:4 (C)	3.9 ⁴	2.2 ³	2.9 ⁴	4.3 ⁵	4.6 ⁴	2.7 ⁴	3.1 ³	4.7 ⁴	3.5 ⁴
20:4	4.4	6.3	6.1	7.6	8.1	7.5	8.1	7.0	6.9
Post 20:4 (D)	0.8 ¹	0.6 ¹	0.8 ¹	0.9 ¹	1.0 ¹	2.9 ³	1.1 ²	1.8 ²	1.2 ²
Total components analyzed	22	21	19	20	15	25	17	18	20

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 8C
FATTY ACID COMPOSITION OF THE CHOLESTERYL ESTER FRACTION HDL₂₊₃ LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Pre 16:0 (A)	4.1 ⁹	2.0 ⁷	2.9 ⁸	1.6 ⁸	2.2 ³	1.4 ⁸	0.7 ⁵	2.5 ⁵	2.2 ⁷
16:0	11.2	10.5	11.1	11.7	12.4	10.9	9.6	10.0	10.9
16:1	3.8	3.0	2.9	3.4	4.8	3.3	2.5	4.7	3.6
16:1-18:0 (B)	0.7 ¹	0.9 ²	0.9 ²	0.6 ²	1.8 ²	0.5 ²	0.4 ²	0.6 ¹	0.8 ²
18:0	1.2	1.1	1.1	1.4	1.6	1.0	1.2	1.4	1.3
18:1	16.6	18.1	17.9	20.7	19.6	21.6	15.4	19.6	18.7
18:2	51.9	55.1	52.8	46.9	43.5	47.8	56.0	44.5	49.8
18:2-20:4 (C)	3.4 ⁴	2.1 ⁴	2.6 ⁴	3.8 ⁵	5.0 ⁴	2.3 ⁴	3.1 ³	4.7 ⁴	3.4 ⁴
20:4	6.1	6.5	6.3	7.9	8.2	8.3	10.4	9.1	7.8
Post 20:4 (D)	1.1 ¹	0.6 ¹	1.4 ²	1.9 ³	0.8 ¹	3.0 ⁵	0.7 ¹	2.9 ³	1.6 ²
Total components analyzed	21	20	22	24	16	25	17	19	21

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 9A
FATTY ACID COMPOSITION OF THE GLYCERIDE-CONTAINING FRACTION S_F
20 TO 10⁵ LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Pre 16:0 (A)	3.5 ¹⁰	4.5 ⁹	7.6 ⁷	3.7 ⁸	3.2 ⁴	2.0 ³	2.1 ⁶	3.4 ⁷	3.7 ⁷
16:0	29.2	25.7	25.0	22.5	25.9	27.0	23.5	25.6	25.5
16:1	3.2	3.9	3.7	6.0	5.0	3.9	3.4	7.0	4.5
16:1-18:0 (B)	1.1 ¹²	1.0 ²	1.0 ²	1.1 ²	1.7 ²	0.9 ²	0.9 ²	0.6 ¹	1.0 ²
18:0	6.7	2.5	3.9	2.4	6.2	7.1	5.2	3.3	4.7
18:1	38.5	30.5	29.2	37.3	40.4	44.3	32.7	35.7	36.1
18:2	15.9	27.3	23.4	21.7	13.7	11.9	22.6	16.9	19.2
18:2-20:4 (C)	1.4 ²	3.0 ⁵	3.9 ⁶	3.9 ⁵	3.2 ⁴	2.1 ⁵	5.3 ⁵	4.5 ⁴	3.4 ⁴
20:4	0.5	1.0	1.6	1.5	0.8	0.8	3.6	1.5	1.4
Post 20:4 (D)	—	0.5 ¹	0.7 ²	—	—	—	0.9 ²	1.6 ¹	0.5 ¹
Total components ana- lyzed	20	23	22	21	16	16	21	19	20

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 9B
FATTY ACID COMPOSITION OF THE GLYCERIDE-CONTAINING FRACTION
S_F 0 TO 20 LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Pre 16:0 (A)	7.9 ¹¹	6.9 ¹⁰	8.2 ⁹	6.1 ⁸	3.3 ⁴	1.3 ⁵	1.8 ⁶	1.5 ⁷	4.6 ⁸
16:0	25.6	27.5	25.5	24.1	24.0	22.9	23.6	27.8	25.1
16:1	4.3	3.5	4.2	5.3	5.1	3.6	3.8	6.1	4.5
16:1-18:0 (B)	2.2 ²	1.4 ²	1.7 ²	1.6 ²	1.9 ²	1.5 ³	0.9 ²	0.6 ¹	1.5 ²
18:0	5.4	3.7	5.6	4.2	5.3	5.3	4.9	4.1	4.8
18:1	35.1	34.3	28.2	38.8	41.1	45.2	34.9	38.0	37.0
18:2	16.0	17.8	19.1	14.1	14.4	12.9	24.0	12.8	16.4
18:2-20:4 (C)	2.7 ⁴	2.3 ⁶	3.4 ⁴	3.8 ⁵	4.0 ⁴	3.3 ⁶	2.8 ⁵	3.9 ⁴	3.3 ⁵
20:4	0.8	1.4	2.1	1.5	1.1	1.4	2.2	1.4	1.5
Post 20:4 (D)	—	1.2 ³	1.8 ³	0.5 ¹	—	2.6 ⁷	1.3 ²	4.0 ²	1.4 ²
Total components ana- lyzed	24	27	24	23	16	27	21	20	23

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 9C
FATTY ACID COMPOSITION OF THE GLYCERIDE-CONTAINING FRACTION
HDL₂₊₃ LIPOPROTEINS

Case.....	1	2	3	4	5	6*	7	8	Mean
Pre 16:0 (A)	5.1 ⁸	5.5 ¹⁰	9.3 ⁸	5.1 ⁹	4.5 ⁵	1.6 ⁵	3.1 ⁷	3.2 ⁶	4.7 ⁷
16:0	30.0	25.1	23.3	22.8	26.1	23.9	23.9	27.2	25.3
16:1	3.9	3.6	3.6	5.1	5.4	3.9	4.0	5.9	4.4
16:1-18:0 (B)	1.9 ²	1.2 ²	2.1 ²	1.2 ²	2.1 ²	1.2 ²	1.2 ²	0.7 ¹	1.4 ²
18:0	5.9	4.3	5.3	2.9	6.1	7.2	4.8	5.7	5.3
18:1	37.6	34.4	27.2	38.2	34.3	43.7	32.0	37.2	35.6
18:2	13.0	20.3	17.6	16.1	15.0	13.5	24.1	12.3	16.5
18:2-20:4 (C)	2.5 ³	2.7 ⁶	5.9 ⁴	5.2 ⁶	4.7 ⁵	2.6 ⁶	2.7 ⁶	5.3 ⁴	4.0 ⁵
20:4	—	1.4	2.3	2.0	1.2	1.4	2.3	1.3	1.5
Post 20:4 (D)	—	1.5 ²	3.6 ³	1.5 ²	0.6 ²	1.0 ³	1.9 ²	1.1 ²	1.4 ²
Total components ana- lyzed	19	26	23	25	20	22	23	19	22

* Methyl ester samples were prepared by microsublimation.¹⁴

TABLE 10A

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION S_f 20 TO 10^5 LIPOPROTEINS

Case.....	1	2	3	4	5	6	7	8	Mean
Pre 16:0 (A)	1.9 ³	3.1 ⁷	2.2 ⁵	2.9 ⁷	1.0 ⁴	1.0 ³	1.3 ⁵	1.6 ³	1.9 ⁶
16:0	37.1	44.4	26.5	40.7	23.2	21.9	22.2	25.8	30.2
16:1	1.3	1.1	1.4	1.5	1.7	0.8	0.9	1.4	1.3
16:1-18:0 (B)	1.4 ²	1.8 ²	1.5 ²	2.0 ²	1.3 ³	0.6 ¹	0.7 ²	0.6 ¹	1.2 ²
18:0	16.3	19.7	14.5	23.2	16.2	16.7	15.4	14.1	17.0
18:1	10.6	12.6	10.2	16.4	12.7	12.5	8.6	14.1	12.2
18:2	20.0	9.6	25.2	7.6	20.8	20.6	23.7	18.9	18.3
18:2-20:4 (C)	5.0 ⁵	2.6 ⁴	4.4 ³	2.7 ⁴	4.9 ⁵	5.2 ⁶	7.0 ⁶	6.5 ⁵	4.8 ⁵
20:4	6.5	0.9	9.4	1.2	12.1	11.6	11.1	9.3	7.7
Post 20:4 (D)	—	4.2 ⁷	4.8 ⁴	1.8 ²	6.3 ⁸	9.3 ⁵	9.2 ⁸	7.7 ⁸	5.4 ⁵
Total components analyzed	21	26	20	21	25	22	27	29	24

TABLE 10B

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION S_f 0 TO 20 LIPOPROTEINS

Case.....	1	2	3	4	5	6	7	8	Mean
Pre 16:0 (A)	2.8 ⁹	2.3 ⁷	4.0 ⁷	3.2 ⁷	1.1 ⁴	0.7 ³	1.2 ⁴	1.2 ⁷	2.1 ⁶
16:0	34.0	28.0	28.7	40.9	24.8	23.0	23.6	27.1	28.8
16:1	1.3	1.1	1.3	1.5	1.7	0.7	0.8	1.3	1.2
16:1-18:0 (B)	1.4 ²	1.1 ²	1.3 ²	1.5 ²	1.6 ²	0.6 ¹	0.6 ²	0.5 ¹	1.1 ³
18:0	13.2	13.1	14.1	22.2	17.0	16.4	15.4	14.0	15.7
18:1	9.4	10.0	10.2	15.8	13.1	12.0	7.8	13.5	11.5
18:2	19.4	24.1	26.3	7.8	20.8	19.2	22.4	17.6	19.7
18:2-20:4 (C)	5.4 ⁵	4.7 ⁴	4.0 ³	2.5 ⁴	5.8 ⁵	5.9 ⁴	6.5 ⁴	6.8 ⁵	5.2 ⁴
20:4	7.4	10.1	9.2	1.3	13.2	10.9	10.5	9.5	9.0
Post 20:4 (D)	5.7 ⁷	5.6 ⁶	0.9 ²	3.4 ⁵	0.8 ²	10.7 ³	11.3 ⁷	8.4 ⁷	5.9 ⁶
Total components analyzed	29	25	20	24	19	22	23	26	24

TABLE 10C

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION HDL₂₊₃ LIPOPROTEINS

Case.....	1	2	3	4	5	6	7	8	Mean
Pre 16:0 (A)	4.4 ³	3.7 ⁷	3.1 ⁷	2.2 ⁷	1.0 ⁴	1.0 ³	1.3 ⁴	1.1 ⁶	2.2 ⁶
16:0	29.1	25.1	25.6	43.7	23.4	21.4	23.7	26.0	27.3
16:1	1.3	1.5	1.3	1.7	1.6	0.9	1.0	1.3	1.3
16:1-18:0 (B)	1.2 ²	1.8 ²	1.6 ²	1.6 ²	1.3 ²	0.7 ¹	0.8 ²	0.4 ¹	1.2 ²
18:0	14.8	11.7	13.8	23.1	15.3	15.9	16.4	13.7	15.6
18:1	10.6	10.1	10.0	16.5	12.2	11.9	8.9	13.1	11.7
18:2	23.1	22.0	27.4	8.0	19.1	19.9	23.1	18.3	20.1
18:2-20:4 (C)	4.9 ⁴	5.8 ⁴	4.3 ³	2.5 ⁴	6.3 ⁴	5.2 ⁵	6.4 ⁵	6.6 ⁵	5.2 ⁴
20:4	10.7	12.1	11.9	0.8	13.6	14.3	11.2	11.8	10.8
Post 20:4 (D)	—	6.4 ⁴	1.1 ²	—	6.3 ⁷	8.9 ⁶	7.3 ⁸	7.6 ⁷	4.7 ⁴
Total components analyzed	20	23	20	19	23	21	25	25	22

cases reflect two of the most extreme lipoprotein abnormalities, yet they do not depart materially from the general fatty acid distributions observed among the other normal individuals studied. If gross fatty acid composition abnormalities were a general feature of disturbances in lipid transport we should expect such abnormalities to be revealed in these two extreme clinical conditions. Thus, considering the fatty acid composition data, it appears more likely that disturbances in lipid metabolism are reflected in elevated concentrations of specific serum lipids such as the glycerides, cholesteryl esters, cholesterol, and the phospholipids. Also, depending upon the relative amounts of these lipids that are present in the blood stream, a specific lipoprotein distribution⁴ may be necessary for their transport.

The above tentative conclusion is based also in part upon the gas-chromatographic studies of James *et al.*,²⁰ in which no gross abnormality in fatty acid composition was detected when the fractionated total serum lipids of coronary patients and normals were compared. Moreover, Hallgren *et al.*²² failed to observe any significant difference between the fatty acid composition of all the major lipid fractions isolated from the serum of normal individuals and diabetic patients.

At least two principle sources of potential error exist in fatty acid composition work such as we have presented. First, the silicic acid fractionation may present difficulties in the separation of the cholesteryl ester fraction from the glyceride-containing fraction. There can be considerable tailing of the cholesteryl ester fraction with the unsaturated cholesteryl esters eluted more slowly than the saturated esters.²² Also there is the possibility, especially where relatively large amounts of glycerides are present in the lipid extract, that the saturated glycerides may be eluted in close proximity to the late-appearing cholesteryl esters.²⁴

Solvent contaminations present another potential source of error. Because these contaminations give components on the gas chromatogram that cannot be distinguished readily from methyl ester components, it is desirable that a solvent blank be run with each group of fatty acid-containing samples.

Conclusions

Although our studies on fatty acid composition of the serum lipids and lipoproteins are preliminary in nature, we can say that, for the serum data together with the available data of other workers, there appears to be a relatively characteristic fatty acid pattern for each fatty acid-containing serum lipid fraction. For all chemical fractions studied the S_f 0 to 20 and HDL₂₊₃ lipoprotein groups appear broadly similar. However, the S_f 20 to 10⁵ lipoprotein data do not show a consistent pattern. The association of the S_f 20 to 10⁵ lipoprotein fraction with the early phases of fat absorption and transport may be responsible in part for these observed chemical differences. Thus cholesteryl esters in the early phase of transport in the blood stream may have a different fatty acid distribution from the cholesterol esters that persist for longer periods of time in the bloodstream as part of the lipid moieties of the S_f 0 to 20 and HDL₂₊₃ lipoproteins. It should be noted that in the average individual the S_f 20 to 10⁵ lipoprotein group usually carries about 70 per cent

of the serum glycerides, but only about 20 per cent of the total serum cholesterol esters. Also the major portion of this S_f 20 to 10^5 lipoprotein group has been found² to be among the lipoprotein classes most significantly elevated in patients with coronary artery disease.

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CERULOPLASMIN IN HEALTH AND DISEASE*

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Ceruloplasmin is a blue alpha-globulin with a molecular weight of 151,000.^{1,2} Each molecule binds 8 atoms of copper, tightly but reversibly,³ and virtually all of the copper in plasma is so bound.^{1,2} The protein is probably synthesized in the liver⁴ and, although primarily a plasma protein, is also found in synovial,⁵ ascitic,² and cerebrospinal fluids⁶ (I. H. Scheinberg, unpublished observations). It is immunochemically specific.⁷ Though it has never proved helpful in understanding the physiology of ceruloplasmin, the moderate oxidase activity of this protein toward a variety of substrates,² of which paraphenylenediamine is the best, provides a useful means of quantitative analysis.^{3,8-14}

In normal human adults there are about 30 mg. of ceruloplasmin per 100 ml. of plasma. In health this concentration is rather constant but it varies with age and in pregnancy.^{8,15-20} The newborn infant possesses about 7 mg. per 100 ml.¹⁷⁻²¹ This concentration rises to reach the adult levels, or exceed it slightly, by about 3 to 6 months of age, and there may be a further slight, gradual increase with old age.²² In the last trimester of pregnancy the concentration of the protein rises two-, or even threefold, and promptly falls to normal within several days of parturition. The simultaneously low neonatal and high maternal concentrations indicate that ceruloplasmin does not cross the placenta freely.

The administration of estrogens can also raise the plasma concentration of ceruloplasmin markedly,^{15,23-27} and it is possible that the elevation seen in pregnancy is mediated through estrogens. The administration of androgens,²⁸ and triiodothyronine²⁹ is reported to increase the concentration of ceruloplasmin and, although this effect is claimed for the phenothiazines and phenobarbital, the evidence is unconvincing.^{30,31}

Like a few other plasma proteins, ceruloplasmin exists in several molecular forms. Thus purified ceruloplasmin, prepared from the plasma of 9109 donors by the methods of Cohn, has been further fractionated chromatographically, and four different ceruloplasmins have been demonstrated.³⁴ These variants of ceruloplasmin are not simply artifacts of the fractionating process since at least two molecular variants of the protein can be isolated from the whole serum of individuals merely by use of the mild methods of column chromatography.^{20,32-34} The various ceruloplasmins can be differentiated from each other by electrophoretic methods³³ which probably reflect differences in charge between the various ceruloplasmins. These charge differences, in turn, may be due to variations in the amino acid content of the polypeptide chains or to differing amounts of other charged components of ceruloplasmin, such as neuraminic acid.²

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The significance of ceruloplasmin's heterogeneity is still to be defined. In our laboratory the proportion of the two principal chromatographic fractions does not seem to differ in any of a number of healthy and sick individuals studied. We do not know yet whether these variants are inherited, acquired or different stages²⁰ in the normal metabolic pathway that ceruloplasmin follows.

Although, therefore, there are no diseased states known in which qualitative differences in ceruloplasmin from normal occur, there are many conditions in which the concentration of the protein varies. A peculiarity of these variations is that, although the significance of an increase in the concentration of ceruloplasmin is not known, we understand something of the nature of decreases in its concentration.

There are a great number, and many types, of pathologic conditions that are associated with increases in the concentration of ceruloplasmin. These include inflammatory conditions, some of which are acute, such as hepatitis³⁵ and pneumonia,¹⁶ and some of which are chronic, such as tuberculosis,¹⁶ silicosis,³⁶ and rheumatoid arthritis;^{9,38,39} myocardial infarction;^{39,40} neoplasms, including such diverse forms as carcinomas,^{39,40} acute and chronic leukemias and lymphomas;^{9,37,38,40} various forms of anemia;^{38,41} the nutritional deficiencies found in pellagra⁴² and chronic alcoholism;⁴⁴ many of the obscure neurological disorders;^{16,44} and perhaps hyperthyroidism.^{45,46} Consequently there is as little specific diagnostic significance in an increased ceruloplasmin concentration as there is in an increased erythrocyte sedimentation rate.

Decreases in the plasma concentration of ceruloplasmin may result from (1) the abnormally low absorption of copper found in tropical⁴⁷ and nontropical, sprue⁴⁸ and in scleroderma of the small bowel (I. Sternlieb, J. C. Brosseau, and H. D. Janowitz, unpublished observations); and from (2) generalized abnormalities in protein metabolism such as those found in some infants with hypoproteinemia, hypoferremia, and anemia,^{49,50} in kwashiorkor,^{51,52} in the renal protein losses of nephrosis,¹⁶ or the intestinal protein losses of hypercatabolic hypoproteinemia or protein-losing enteropathy⁵³ (Sternlieb, Brosseau, and Janowitz, unpublished observations). In none of these conditions are there any ill effects that can be ascribed to ceruloplasmin deficiency and, in all of them, the deficiency of ceruloplasmin results from the deficiency in the copper and/or protein moieties of ceruloplasmin that the disease has brought about.

Hereditary deficiency of ceruloplasmin contrasts sharply to the foregoing since fatal pathologic changes are ultimately associated with this inherited abnormality, which is also to say that hereditary deficiency of ceruloplasmin is not the result of a disease.

These pathologic changes define Wilson's disease and appear to be synonymous with human chronic copper toxicity for the following reasons. Although almost every patient has a deficiency or absence of ceruloplasmin,^{6,7,9,16,17,20,25,44,54,55} he also has—somewhat paradoxically—an excess of copper in virtually all other tissues.⁵⁶⁻⁵⁸ This may be seen in the corneal Kayser-Fleischer rings, which are due to copper deposited in Descemet's membrane,⁵⁹ and can be demonstrated in other tissues by chemical analysis.^{16,56,58,60} Early in life these deposits are unassociated with pathologic changes but, eventually severe

histologic and functional damage appears, predominantly in liver^{60,61} and brain.^{62,63} If absorption of copper is minimized and its excretion is promoted by pharmacologic means in these patients, the deposits in the eye may disappear and considerable improvement in the neurologic disease may result.^{64,65}

It is remarkable that everybody does not develop Wilson's disease, for almost all of us ingest more than 5 per cent of our total body content of copper every day. Our needs for copper are so modest that, although we retain essentially none of this ingested copper, we never suffer from deficiency of copper.⁶⁶ Furthermore, in the face of this dietary surfeit we do not suffer from copper toxicity presumably because of a mechanism that prevents the copper in food and drink from being retained in amounts that are toxic. Patients with Wilson's disease must lack this mechanism, for they ingest no more copper than normal subjects. Since these patients also lack normal amounts of ceruloplasmin, it seems reasonable to conclude that this protein plays an important role in a normal mechanism regulating the retention of ingested copper.

There are several facts that conflict with this theory. First, neither the magnitude of the tissue copper deposits, the patient's age at onset of clinical disease, nor the severity of the disease have been correlated with the degree of ceruloplasmin deficiency.^{67,68} Second, there are rare patients who have indubitable Wilson's disease despite a normal concentration of ceruloplasmin.^{69,70} Third, there are some individuals who have inherited low concentrations of ceruloplasmin and who do not have Wilson's disease.⁷¹

The magnitude of copper deposits, the age of onset, and the severity may all depend on the actual intake of copper, and this can vary widely in individuals. Second, a review of those patients with Wilson's disease in whom normal concentrations of ceruloplasmin are found shows that the normal concentration has occurred when overt hepatic decompensation is present. Thus when normal concentrations of ceruloplasmin are observed in patients with Wilson's disease, they may be the consequence of inflammation or necrosis of the liver or of the effects of the abnormal metabolism of estrogens that may accompany hepatic dysfunction. Third, there is still no evidence to show how prolonged is the deficiency of ceruloplasmin, which is rarely found in the asymptomatic heterozygous carriers of a single dose of the abnormal gene.

Regardless of the validity of our theoretical considerations, a determination of the concentration of ceruloplasmin in serum remains, in our opinion, the single most important criterion by which to establish the diagnosis of Wilson's disease. Conversely, to diagnose this disease is the principal clinical value of determining the concentration of ceruloplasmin. TABLE 1 lists the ranges of concentrations of ceruloplasmin found in the sera of 75 patients with Wilson's disease in our laboratory. From these figures it is apparent that 84 per cent of these 75 patients have ceruloplasmin concentrations of less than 10 mg. per 100 ml. of serum, whereas normal subjects rarely have less than 20 mg. per cent. Most important, ceruloplasmin deficiency has been found as the first sign of Wilson's disease in 10 of these patients.

However ceruloplasmin determination cannot always confirm, or rule out, the diagnosis of Wilson's disease, because of the rare patients in whom the concentration of ceruloplasmin is not different from normal (TABLE 1). In

patients of this sort we must determine whether other manifestations of Wilson's disease can be demonstrated. These are the presence of Kayser-Fleischer rings, which can sometimes be seen only with a slit lamp, the excretion of more than 100 μg . of urinary copper per day or, most valuable, proof that excess copper has been deposited in tissues by showing that there are more than 150 μg . of copper per gram of dry liver. To our knowledge no other disease is associated with any of these three findings or with persistent deficiency of ceruloplasmin. If two or more of the four are present in any individual, the diagnosis of Wilson's disease is virtually certain.

We may emphasize that simple screening tests are available for the detection of deficiencies in serum ceruloplasmin,^{10,72} so that the diagnosis of Wilson's disease can be made with considerable certainty in infants and children who are still asymptomatic and in whom the beneficial—or, rather, the prophy-

TABLE 1
DISTRIBUTION OF CONCENTRATIONS OF CERULOPLASMIN IN SERUM OF 75
PATIENTS WITH WILSON'S DISEASE

Ceruloplasmin (mg. %)	Patients	
	No.	Per cent
0- <1	22	29.3
1-4.9	19	25.3
5-9.9	22	29.3
10-14.9	6	8.2
15-19.9	4	5.3
>20	2*	2.6
	Total 75	100.0

Normal: 20-35 mg. %

* One of these patients is R.M. of Sass-Kortsak *et al.*⁶⁹

lactic—effects of therapy should be maximal. A screening test of this sort probably should be applied once in his lifetime to every child.

We believe that ceruloplasmin is intimately concerned with the regulation of copper metabolism, by a mechanism still unknown. It may be simply that ceruloplasmin provides a large molecule that holds copper in the blood and prevents its relatively free diffusion into tissues.²⁷ Ultimate destruction of a ceruloplasmin molecule may be associated with the release of its copper for excretion via bile or the intestinal wall.

Summary

Ceruloplasmin, which exists in several molecular variants, may rise in concentration inexplicably in many diseases and in pregnancy. Its concentration is low in the neonate, in the absence of sufficient copper or protein synthesis in the body, and in conditions where there is renal or intestinal loss of ceruloplasmin. However, the most important cause of alterations in its concentration is that which is inherited. This permits us to diagnose a fatal disease

while the patient-to-be is still asymptomatic, and study of this deficiency has provided whatever knowledge we have of ceruloplasmin's physiologic function.

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EXPERIMENTAL AND EVOLUTIONARY SIGNIFICANCE OF SIMILARITIES AMONG SERUM PROTEIN ANTIGENS OF MAN AND THE LOWER PRIMATES

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The remarkable similarities observed in the protein complement of blood sera of mammals and the intimate relationship of its constituents to physiology, both normal and abnormal, have made its investigation one of the most engaging of biochemical research. The more intensive the investigation, however, and the more discriminating the tools of the investigator, the more striking and meaningful do the differences appear to become.

Immunochemical techniques such as immunoelectrophoresis are proving extremely useful when a problem requires the detection of similarities and differences among complex mixtures of macromolecules. Among such mixtures, human serum proteins in their normal and pathological expression, have received by far the most attention¹⁻⁴ since the introduction of the immunoelectrophoretic technique.⁵ If the enormous fund of information about this mixture of proteins could be directly transferable to closely related species, research involving serum proteins in these animals would be significantly advanced.

The use of antisera against the antigens of one species or strain to examine the antigens of another is not a new concept. Some systems of classification of bacterial strains are based upon the relative specificity of heterologous reactions, but this is only one of the applications of systematic serology. Very few studies, however, have employed immunoelectrophoresis to compare antigenic relationships among living forms, and those that have,^{6,7} with the exception of one,⁸ were not devoted to several members of closely related systematic groups.

The general plan of this study was to obtain immunoelectrophoretic patterns for a number of primate species, representing the three superfamilies of the suborder Anthropeidea. Although possible applications of this approach to experimental medicine and biology are pointed out, our primary object was to assess the observations and data in terms of primate evolution and systematics.

Materials and Methods

Immunelectrophoretic analysis was performed by the micromethod, a modification introduced by Scheidegger.⁹ An Agafor apparatus* designed for microelectrophoresis in gels was employed. Electrophoresis was carried

* Agafor: trademark for a Swiss apparatus distributed by National Instruments Laboratories, Washington, D.C.

out in 1.5 per cent Difco Noble Agar in veronal buffer, pH 8.2, ionic strength 0.05, for 1 hour at a voltage gradient of 4 volts/cm. Immunoelectrophoretic patterns were obtained by allowing diffusion and precipitation to take place at $4^{\circ}C$. This provided uniform conditions and prevented overdevelopment frequently obtained by overnight diffusion at room temperatures. Development time was usually 24 hours, although some experiments required longer periods. The developed slides were washed for 2 days in 0.3 per cent saline, then 2 days in distilled water before drying. The patterns were stained with bromphenol blue according to Uriel and Scheidegger¹⁰ and washed in 10 per cent acetic acid. Photographs were made by means of a photoenlarger using the slide as a negative.

Antigens consisted of samples of aged and fresh human serum, aged and fresh rhesus serum obtained through the courtesy of Max Theiler of the Rockefeller Foundation Virus Laboratory, New York, N.Y., and various primate sera received from the Serological Museum, Rutgers University, New Brunswick, N.J., through the courtesy of Douglas Gemeroy. The latter may not have been normal specimens since they were originally obtained from zoo exhibits, probably when the animals were being treated for disease.

Antisera included: S-31, horse antihuman plasma from the Pasteur Institute, Paris, France; S-438, rabbit antihuman macroglobulin from Henry Kunkel; and S-134, rabbit antihuman A-anti-A substance specific precipitate from Elvin Kabat. Several other antisera were employed in the course of these studies, but only those mentioned specifically are dealt with in this presentation.

Experimental Results

A normal human serum pattern was obtained with relative quantities of antigen and antiserum S-31 in such ratio that the γ -globulin reaction line was in approximate equivalence. This meant that less human serum than customary was employed. While this permitted the development of most of the pattern before the albumin reaction had disappeared due to huge excess of antigen, it did preclude the development of numerous precipitates of minor α and β globulins. Most of the experimentally identified constituents could nevertheless be recognized and labeled (FIGURE 1) to provide orientation for the comparative study. These identifications have been made by many different investigators whose specific problems may not have been concerned with more than one or two individual proteins.

The human pattern from FIGURE 1 is presented in FIGURE 2 as the type exhibit for comparative purposes. All primate patterns were produced with the same relative amounts of serum and antiserum. The patterns have been arranged from top to bottom according to their presumed relationship to man. Progressing from the closer relatives to the more distantly classified Primates, several conclusions are reached at a glance. Albumin and γ globulin are readily identified homologues. Transferrin, except in mandrill and capuchin monkey, is also distinguishable partly by its mobility and partly by the position between sources of antigen and antibody at which the precipitate forms. There is a decrease in density of precipitates moving down the family

Albumin
 α_1 Lipoprotein
 α_1 Glycoprotein
 Gc protein
 α_2 Haptoglobin
 α_2 Macroglobulin
 Ceruloplasmin
 β_1 Lipoprotein
 β_1 Transferrin
 β_2 -A globulin
 $(\beta_2(\gamma))$ Macroglobulin
 γ Globulin

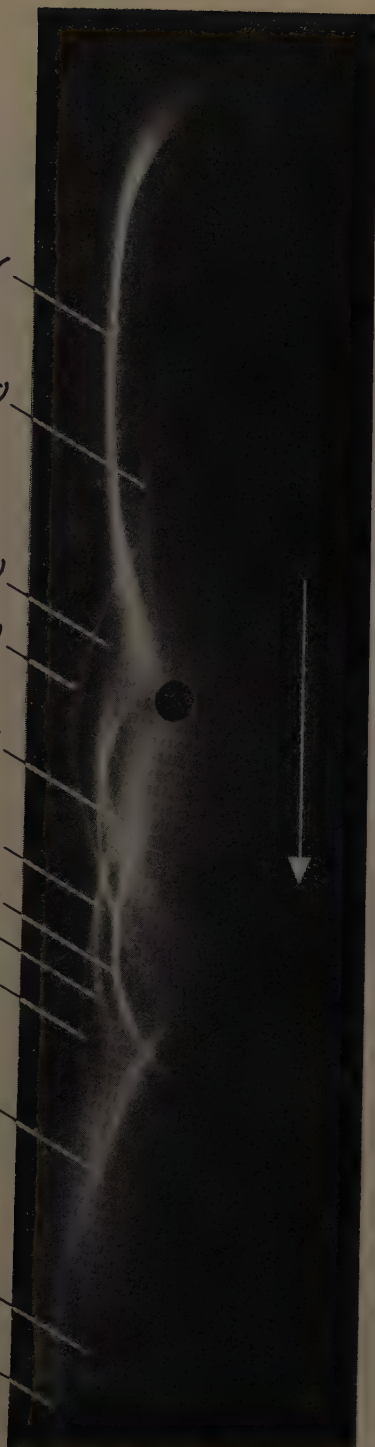


FIGURE 1. Normal immunoelectrophoretic pattern of human serum developed with hyperimmune horse antiserum S-31. Relative quantities of human serum to antiserum were selected to provide optimum visibility of identifiable reaction lines.

tree; but this is unreliable in certain cases where the relative concentration of a protein homologue in a heterologous serum differs significantly.* For ex-

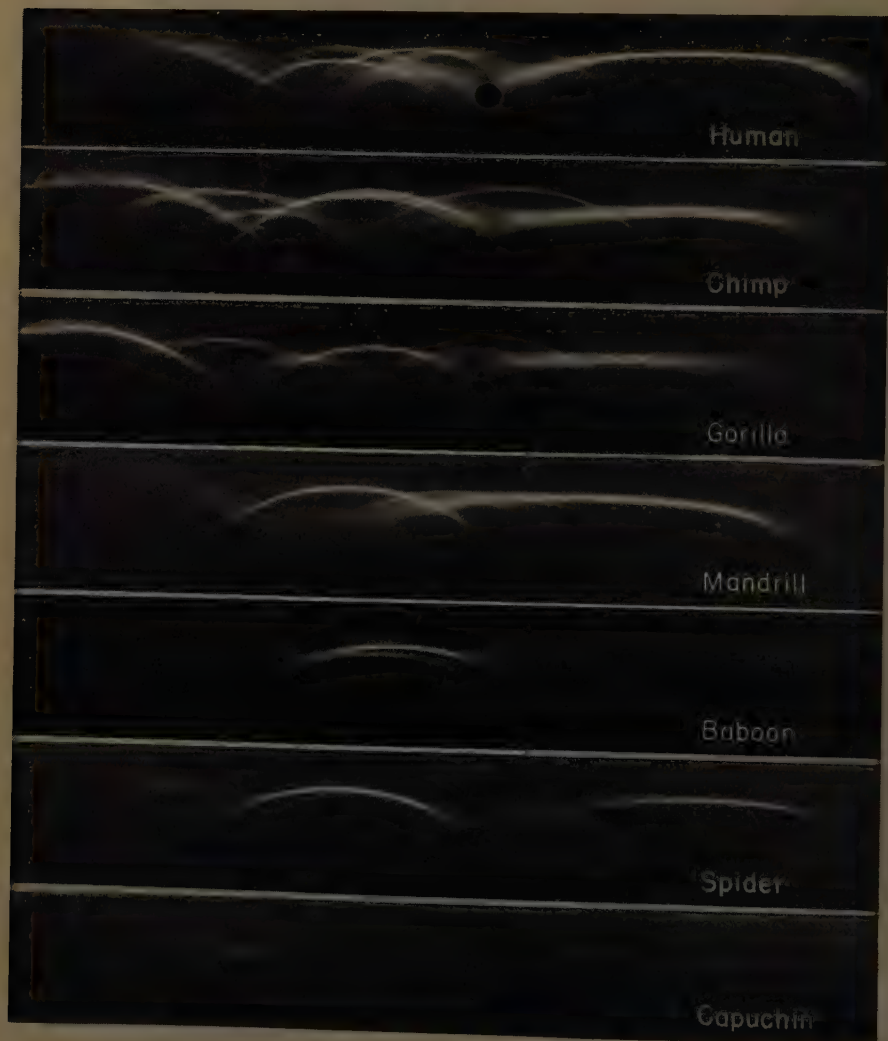


FIGURE 2. Primate serum patterns developed with horse antiserum S-31. The panels are arranged from top to bottom according to their systematic relationship to man. See FIGURE 7.

* In immunogenetics the serological term "homologous", meaning same antigen or same source of antigen, is likely to become confused with the purely genetic meaning of the term. In the latter case, homology refers to structures and traits governed by genetic mechanisms interacting in the same manner and related by descent. For a fuller discussion the reader is referred to reviews by Alan Boyden.^{11a and b} In this paper, homologous, homology, and homologue are all used in the genetic sense. The serological term "heterologous" is used to indicate different source of antigen and should not be interpreted as the antonym of homologous.

ample, gorilla albumin appears less in excess than a human sample; therefore a denser precipitate is present at the time development of the pattern was arrested. Horse antibody precipitates are known to be sensitive to excess antigen and, to some extent, to excess antibody. Generally speaking, however, there are fewer and fewer strong reactions in the patterns as the systematic relationships become more remote.

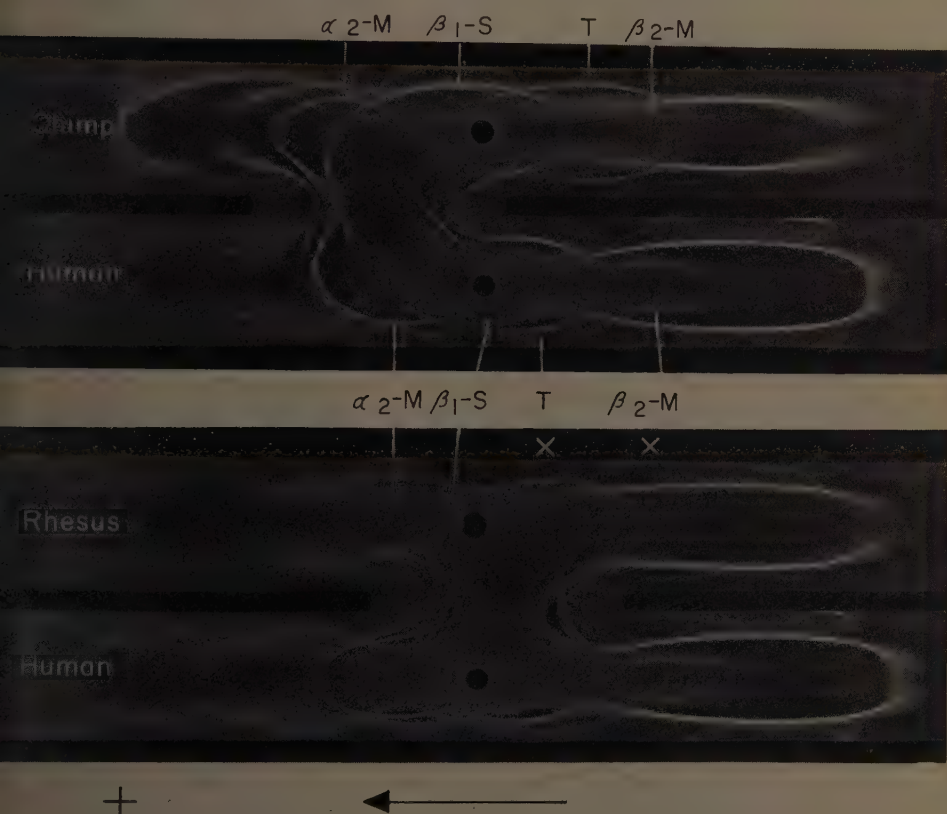


FIGURE 3. Test for homologous proteins in heterologous patterns. Diffusion "gates" in the center antiserum trough may be placed in different position depending on the components to be identified. Undetectable reaction lines in the rhesus pattern are indicated by X.

Even among "normal" humans α and β globulins vary with respect to relative concentration and electrophoretic mobility. Identification of homologues therefore demands more than casual observation. Two types of experiment were employed to establish homology of these serum proteins. FIGURE 3 illustrates a procedure in which complete parallel patterns are developed with S-31 diffusing from the lateral outside troughs. The center trough is interrupted by a "gate" of intact agar through which antigens from both serum samples can diffuse. When and if lines of precipitate join in this region, homology is indicated by reactions of "identity" and "partial identity" as defined by Ouchterlony.¹² Strictly speaking, a line originating in a heterologous

pattern which on further development joins a line in the specific type pattern does so in a reaction of partial identity. The partial fusion or the nonfusion of the lines, however, is sufficient evidence to establish the homology or non-homology of antigens in the heterologous patterns. FIGURE 4 presents another method in which the center trough is omitted and the serum samples are subjected to electrophoresis side-by-side. These experiments, unlike those pictured in FIGURE 2, were performed in relative antigen excess and the diffusion phase was extended up to 4 days. This procedure produced far more complex patterns and assured that reactions of greater intensity would develop. Many antigen homologues could be pointed out in human, chimp, and rhesus serum, some similar in appearance and mobility and some dissimilar in appearance and/or mobility. We shall confine ourselves to but a few to illustrate the power and usefulness of these techniques.

α_2 -Macroglobulin (α_2 -M) has a higher mobility in chimpanzee and a lower mobility in rhesus than in human. β_1 -S* is far more concentrated in chimpanzee than in rhesus or human but in both chimpanzee and rhesus it shows a higher mobility. Transferrin has a lower mobility in chimpanzee than in human. Transferrin in the rhesus is not the reaction first suspected, arrow 1 in FIGURE 4, but the weaker line indicated by arrow 2. Arrows 3, 4, and 5 point to precipitates formed by human antigens diffusing into the rhesus side of the plate, reacting with antibody diffusing through the rhesus antigens. These lines represent reactions for which there are either no antigenic determinants in the rhesus or for which the determinants have a low degree of antigenic correspondence. If there had been determinants of high correspondence, the antibodies would have been bound and hence incapable of diffusing through the array of rhesus proteins. Respectively, these lines represent β_2 -A, β_2 -M, and some antigenic determinant(s) of γ -globulin.

Two rabbit antihuman γ -globulin sera were used to compare the γ -globulin components of various primate sera in antigen excess. Some results of this study are shown in FIGURE 5. The upper pattern of each panel was obtained with S-134 prepared in rabbits by injection of a washed specific precipitate of A substance and human anti-A substance. The lower pattern of each panel was obtained with S-438, rabbit antiserum prepared against human β_2 -macroglobulin.

S-134 produces a single clear line with human β_2 -M and two partially superimposed lines with the γ -globulin. The pattern is the same, though less dense with chimpanzee. β_2 -M is not detected in gorilla and rhesus sera and but faintly in spider monkey. The β -globulin reactions are quite faint with rhesus and spider monkey sera. S-438 gives two reactions with human β_2 -M, one of which is quite strong but is weak or nonexistent in other species. This would indicate either a highly specific antibody, or an antigenic determinant on human β_2 -M that is highly characteristic of the species. There appear to be at least three reactions between S-438 antibodies and γ -globulin. These lines, presumably due to antibodies specific for different antigenic determinants, differ in relative intensity, certain ones decreasing in density more than others as more and more distant relatives are examined. One is tempted

* Tentative nomenclature of the authors. See discussion.

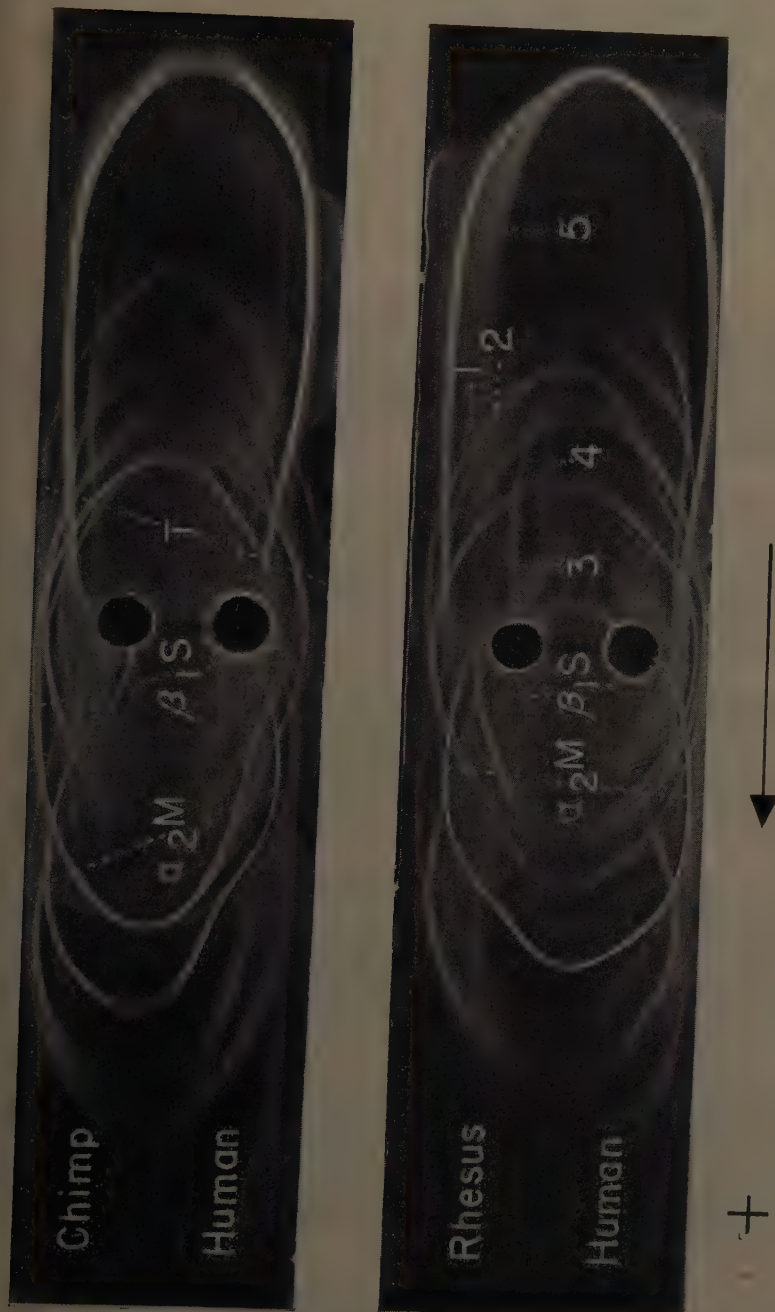


FIGURE 4. Test for homologous proteins in heterologous patterns by omission of center trough. Note mobility differences of labelled homologues. Reaction 1 might be assumed to represent transferrin (T) were it not for its cross reaction with another antigen in the human pattern. Line 2 has been identified as rhesus transferrin. Reactions 3, 4, and 5 are those of human β_2 -A, β_2 -M, and γ -globulin respectively, precipitating with anti-

to conclude that some antigenic determinants of protein homologues have been conservative and some have been more or less radical with respect to evolutionary change.

While multiple line formation with γ -globulin and certain antisera has been described and discussed in terms of dissociation of γ -globulin complexes of

x Rabbit Anti-human γ -Globulin
Antigen Excess

134



+



438

FIGURE 5. Primate γ -globulins reacted with two different rabbit antisera. S-134 developed the upper pattern of each panel, and S-438 developed the lower patterns.

distinct antigenic characteristics by Williams and Grabar,¹³ it was brought into sharp focus by Edelman *et al.*,¹⁴ who demonstrated that papain-treated human γ -globulin migrated with two distinct electrophoretic mobilities, each component possessing distinct antigenic determinants. Edelman *et al.* have called these components S and F for slow and fast mobility. The outer line nearer the trough containing S-438 has been identified as the F-anti-F reaction, and the inner complex of reactions as that due to the precipitation of anti-S antibodies with the S component. In antigen-antibody equivalence it ap-

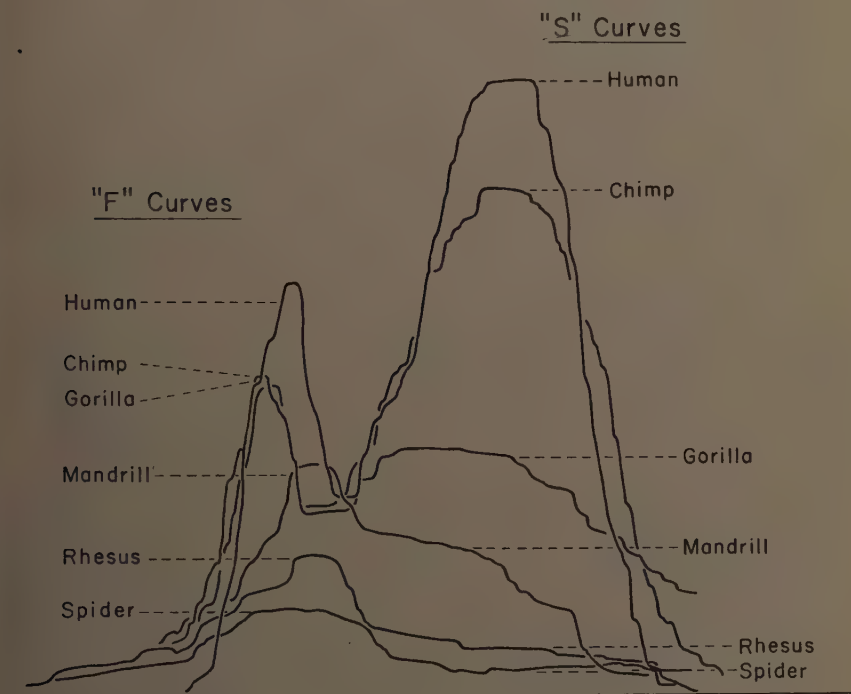


FIGURE 6. Density curves of multiple line precipitates with S-438 were obtained from photographic plates using a double-beam recording microdensitometer.

peared that the density of F-anti-F precipitate was relatively higher in reactions with sera of the lower Primates and was present even when the S-anti-S line might be nearly invisible.

It was of some interest to translate these observations into something approaching quantitative data. Accordingly, transparent photographic plates were made under scrupulously uniform conditions of exposure and development. These were passed through a double-beam recording microdensitometer (Joyce-Loebl Mark III). In this way relative density curves of the precipitate lines were expanded. The tracings for several species are superimposed in FIGURE 6. Analysis of the curves by resolution of peaks and determination of curve areas permitted a calculation of fractional cross-reactivity of the

primate F and S components relative to the specific human-antihuman reactions. The results are presented in TABLE 1. To demonstrate that the disparity of S and F cross-reactivity was not due purely to different relative concentrations of antigen and antibody, 4-times diluted human serum was included as a control. The total density was reduced by about one half but the F reaction in this case was weaker than the S reaction, unlike the full strength heterologous reactions. Included in this table are summarized results obtained by Gerneroy^{15a&b,18} using various antihuman γ -globulin sera and Primate sera in liquid medium, a method described and developed by Boyden and his colleagues.¹⁶⁻¹⁸ Agreement of these data with the S curve fractional values is fairly good with the exception of chimpanzee. Our data for the chimpanzee are high, because of a higher concentration of γ -globulin in the chimpanzee sample due to a prior immunization.

TABLE 1
RELATIVE CURVE AREAS OF HETEROLOGOUS REACTIONS*

	Gel medium (serum 438)			Liquid medium (Gerneroy)†
	S	F	T†	
Human	1.00	1.00	1.00	1.00
Chimp	0.91	0.80	0.90	0.70-0.75
Gorilla	0.57	0.74	0.63	0.62-0.75
Mandrill	0.22	0.51	0.32	0.21
Rhesus	0.09	0.36	0.18	0.14-0.20
Spider	0.05	0.23	0.12	0.00-0.04
Capuchin				0.10
Human/4	0.58	0.50	0.56	

* Relative curve areas of heterologous reactions were obtained by assigning an arbitrary value of 1.00 to human-antihuman curve areas.

† In the T column are relative total curve areas; S and F head columns for relative fractional curve areas contributed by determinants of γ -globulin characteristic of the S and F components described by Edelman *et al.* (14).

‡ Data represent a summary of results of Gerneroy¹⁵ (unpublished work also) obtained by turbidimetric analysis using various antihuman γ -globulin antisera.

Discussion

The immunoelectrophoretic patterns of certain primate sera developed by antiserum to human serum proteins closely resemble the human pattern considered in its range of normal variation. These studies depend on antibodies to common structural groups possessed by protein homologues, in turn produced by cellular homologues, and presumably serving analogous functions. The many applications of these methods and the principles of cross-reactivity to experimental biology and medicine are apparent. This is particularly true in the case of the Primates since their members, notably chimpanzee and rhesus, are used in research almost solely because of their close relationship to man.

Some of the differences noted in the comparative study pictured in FIGURES 2, 3, and 4 are questionable, but in an intriguing way. The Primate samples were obtained originally from zoos and were more than likely drawn when the

animal was under clinical observation for some pathological condition. Indeed the only samples we could affirm to be normal were human and rhesus. Preserved serum from human and rhesus were indistinguishable from the fresh samples, and it was concluded that only slight differences could be attributed to the fact that all other Primate samples had been in storage for various lengths of time. Except for human, rhesus, and perhaps capuchin monkey, however, all samples showed the strong reaction line labeled β_1 -S on FIGURES 3 and 4. In mice with acute bacterial infections we have observed just such a component, which in normal mice appears to be a minor constituent.¹⁹ This component has been identified as haptoglobin both in mice and primates.*

Immunochemistry and Genetics

Specificities of antibodies for antigenic determinant groups are always relative. The more or less closely related two antigenic determinants are, the more or less they will approximate one another in their ability to combine firmly with an antibody made against one of the two. Thus cross-reactions between one specific antibody and its corresponding antigenic determinant on antigen homologues from related species are subject to accurate quantitative measurement. In most immunochemical studies, however, there is no assurance that a reaction between only one antibody and one determinant group is being investigated. More and more experiments are being reported demonstrating that antisera to even a highly purified antigen contain several antibodies, all precipitable by the antigen but specific for different antigenic determinant groups.

Determinant groups of a native antigen represent the expression of gene action on the structure of a macromolecule. That there are similar antigenic determinants on antigen homologues from closely related species suggests that they are governed by genes descended from a common ancestral genome. It is more than likely that alterations of antigenic determinants are initiated not only by the mutation of genes directly governing the chemical make-up of the site, but also by their interaction with other genes in determining the spacial relationships on the surface of a protein molecule. Thus while one might speak with some assurance of whole antigen homologues, it would be difficult or impossible in most cases to determine whether the changes in a homologous antigenic determinant group are the expressions of homologous alleles. There is no reason to assume, moreover, that all genes in any given species have been modified at the same rate during the evolutionary process. Thus it might be expected that certain antigenic determinants, viewed as genetically controlled characteristics of a species, would indicate conservative evolutionary change, while others would give evidence of more radical modification. It follows that two antisera to a given antigen that react with antigen homologues from different species may contain antibodies to different determinants that have differed in their rates of evolutionary change. Semiquantitative data describing cross-reactivity with different antisera may place the

* Williams, C. A., Jr. and C. T. Wemyss, Jr. 1961. Changes produced in mouse plasma proteins by acute bacterial infections. In preparation.

species in the same relative systematic positions but provide widely disparate determinations of immunochemical specificity.

The reactions pictured in FIGURE 5, with Primate γ -globulins and two different antisera are a case in point. S-134 may place the species examined in the same order of relationship to man as S-438, but the semiquantitative data would indicate that rhesus and spider monkeys were relatively more distant from man and the higher apes than data obtained with S-438 would suggest. S-438, however, since it seems to contain a broader spectrum of distinct antibodies, may yield data more consistent with the facts of primate evolution. The ideal evaluation would have to be made with a series of antisera, each containing antibody for only one antigenic determinant, by establishing the relative specificity of each for their determinant homologues at antigen-antibody equivalence. The fact that several reactions are visible in the S-438 pattern attests to the fact that the relative concentrations of antibody and antigenic determinant in these experiments vary considerably.

It is difficult therefore to assess the significance of the differential decrease of cross-reactivity between the F-anti-F and the S-anti-S reactions demonstrated by the methods employed. There are also other imponderables, peculiar to the formation of double lines in agar diffusion studies, that could affect their relative densities. More dense or less dense, however, the F and S reactions place the species examined in the same systematic order, which is a strong indication of the parallel evolution of different antigenic determinants. Of course, such studies might be more profitably conducted on proteins other than γ -globulin, particularly when the state of health of the donor is in question; γ -globulin has become a generic term for a related but heterogeneous group of proteins associated with immune function. A better antigen might be serum albumin, which also has at least three determinants.²⁰ The best antigen, naturally, would be a protein the structure of which has been largely established.

These experiments have served to pinpoint several areas of methodological difficulties in what is now being called immunogenetics. Some are conceptual, some are technical, but none appears insurmountable even at this early stage. Consequently we find it difficult to resist correlating our findings with what is known and surmised about the course of Primate evolution.

Antigenic Correspondence and Evolution

The Primates have attracted more attention in this regard than any other order of mammals, but it is not because of this alone that more is known of their family tree. The work of the paleontologists and taxonomists has been greatly abetted by the fact that modern living forms reflect the entire course of Primate evolution from the most primitive to the most advanced;²¹ hence extinct ancestral forms are more easily classified. We have constructed a partial tree including only those forms that we have studied (FIGURE 7). The placement of the branches of the tree must be considered as our interpretation of the consensus of authority concerning the times of divergence of the ancestors of modern species.* The scale on the right therefore refers only to the

* Our conclusions are based largely on discussions of the systematics and evolution of the Primates in *The Antecedents of Man*, by W. E. Le Gros Clark.²¹

time of branching from the main stem. The living forms have been spaced to correspond to their position on the cross-reactivity scale on the left. In no case, although there was variation within a group, did a member overlap into a cross-reactivity range of another group. Thus it can be asserted that relative biochemical correspondence among these species parallels the systematic relationship provided by morphological characteristics. It must be remembered, however, that the antigenic determinants being compared are traits of modern forms, and that they cross-react with antihuman antibodies only to the extent that both they and the human determinants share features of the more general

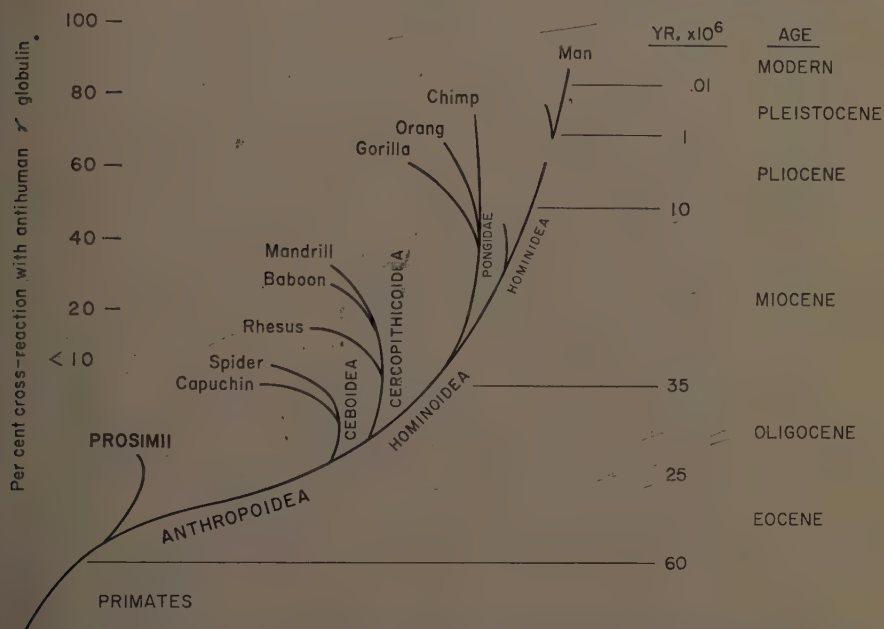


FIGURE 7. Evolutionary tree of the Primates. The time scale on right refers only to the major branches from the main stem. The living forms from man to spider monkey are placed vertically to indicate their approximate cross-reactivity with anti- γ -globulin serum on the scale at the left.

traits characteristic of the group at the time of their branching from the main stem of the tree. Variations within the groups must then be due to evolutionary modification since that time. Changes have undoubtedly occurred during the evolution of the hominids also, but there is no way to establish a range of variation, all forms but one being extinct.

Almost every characteristic of a species will retain some feature of the generic trait and, in turn, of the familial and ordinal traits. While this principle has permitted systematic relationships to be defined, the distance of relationship of one species to another can be expressed only in terms of the time that has elapsed since their divergence from their nearest common ancestor. If distance of relationship were possible to express in terms of immunochemical correspondence of antigen homologues, a concept implicit in the predictions of

Landsteiner²² and in the extensive contributions of Boyden,¹⁶⁻¹⁸ there should be a significant correlation between relative antigenic correspondence and geologic time. Not enough data have been provided by the present study to establish this relationship beyond question, but plots of both S and F component cross-reaction values, and the cross-reaction values found by Gemeroy, suggest strongly that such a direct correlation exists. FIGURE 8 illustrates the plots of the cross-reaction ranges of the major groups of Primates against the

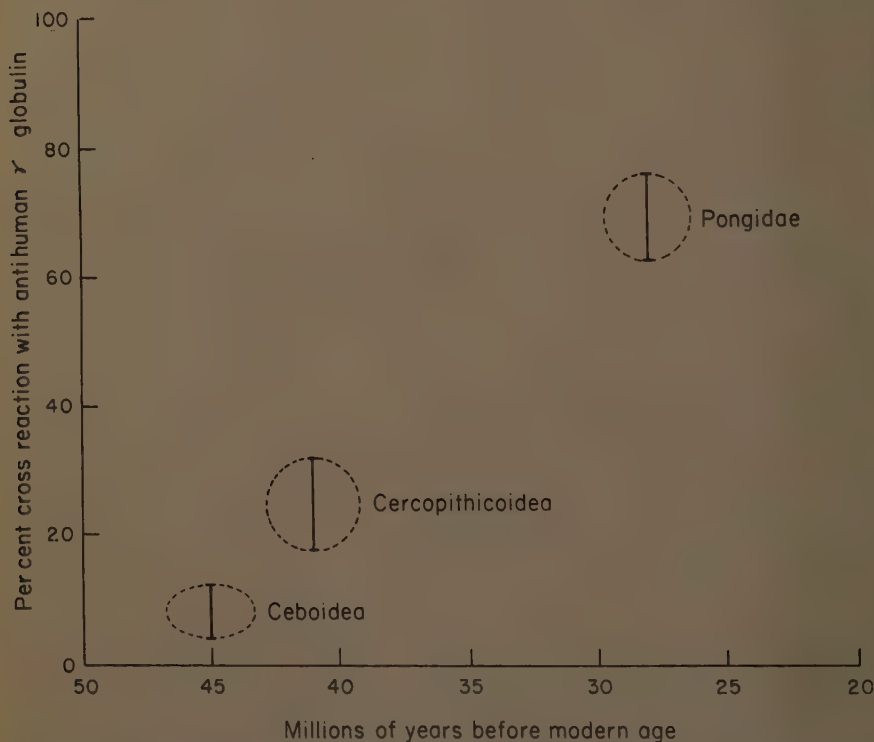


FIGURE 8. Plots of cross-reactivity ranges for the major groups of Primates against elapsed time since their divergence from the main stem of the evolutionary tree.

probable time of the emergence of these groups. We must await further experiment and refinement of techniques, however, to confirm and extend our findings. Furthermore, the validity of a straight-line relationship depends upon the agreement of the paleontologists with our interpretation of their findings; although a range for disagreement is provided by area plots.

An explanation for a straight-line relationship must take into consideration (1) the various evolutionary mechanisms operating to produce ranges of variation in specific traits among surviving forms, (2) the nature of the traits under investigation, and (3) their possible importance to the survival of the species. It is our conclusion that the most probable explanation for this

phenomenon is that, for each genetic factor influencing the expression of antigenic determinants, there has been a relatively uniform mutation rate throughout the period of primate evolution, and that natural selection, while influencing the range of antigenic correspondence among homologous determinants, would not significantly affect the straight-line relationship to time.

Acknowledgments

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Part II. Abnormal Components of Plasma in Disease

IMMUNOELECTROPHORETIC CHARACTERIZATION OF THE SERUM AND URINARY PROTEINS IN PLASMA CELL MYELOMA AND WALDENSTRÖM'S MACROGLOBULINEMIA*

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Immunoelectrophoretic analysis of the serum and urinary proteins of patients with plasma cell myeloma and primary Waldenström's macroglobulinemia has assisted greatly in the elucidation of the interrelationships of these paraproteins to each other and to the normal immunoglobulins. The purpose of this presentation is to review these studies.

Background

At the outset, a definition of certain terms would seem in order. The term immunoglobulins was originally suggested by Heremans¹ as an inclusive designation for the three groups of normal human serum proteins known to possess antibody activity. As schematically presented in FIGURE 1, these three groups or families of immune globulins are: (1) the major gamma fraction (syn. γ_2 , 7S γ) containing the majority of the acquired antibacterial and antiviral antibodies; (2) the beta 2A fraction (syn. β_x), distinguished from the major gamma fraction by virtue of a higher content of conjugated carbohydrate and solubility in 0.025 M ZnSO₄; and (3) the high molecular weight antibody fraction, beta 2M (syn. γ_1 , 19S γ , γ macroglobulin, β_2 macroglobulin). The designations beta 2A and beta 2M were introduced by Grabar and his associates and refer to the immunoelectrophoretic characteristics of these fractions. It is established, however, that the constituents of all three immunoglobulin fractions span a broad range of electrophoretic mobilities, extending from the slow gamma through the beta and into the alpha₂ region, thereby limiting the usefulness of a nomenclature based solely upon electrophoretic mobility.† Until a more satisfactory system is devised, however, this standard of reference will have to be employed.

FIGURE 1 also suggests the currently held hypothesis that each of the three immunoglobulin families is itself comprised of an indeterminate number of

* The work described in this paper was supported in part by Grant CY-2332 from the National Cancer Institute, Public Health Service, Bethesda, Md.

† Recently Heremans² has himself criticized the term "immunoglobulins" on the grounds that this designation should properly include the constituents of the complement and properdin systems. Heremans has suggested that the designation "gamma system" be employed to denote exclusively the three groups of acquired antibody proteins, viz., γ , β_2A , and β_2M . We fail to see particular merit in the latter suggestion, since there may likewise be confusion regarding the members of the "gamma system" possessing electrophoretic mobilities in the beta and alpha₂ ranges, and we shall therefore continue to employ the inclusive term "immunoglobulins" in the manner originally suggested by Heremans¹ as encompassing major γ , β_2A , and β_2M .

individual proteins which, while sharing several physical, chemical, and immunological properties, still retain individual biological specificities. The biological specificities of these individual proteins must, indeed, reside in specific physicochemical and immunochemical properties, but current knowledge of the molecular basis of biological specificity is still quite incomplete. It is evident, however, from the fact that individual immunoglobulins vary in

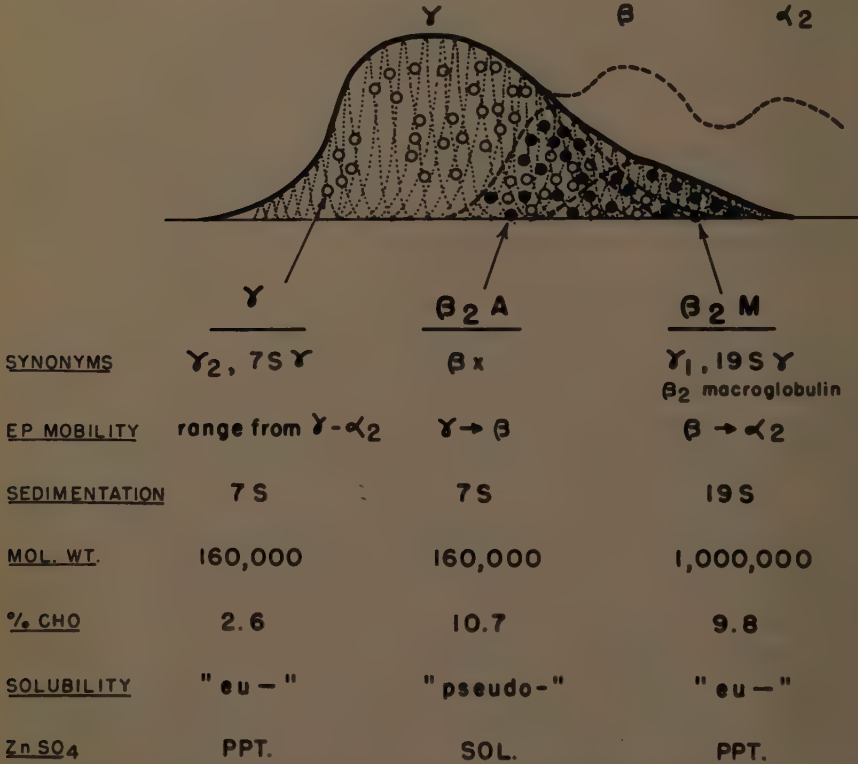


FIGURE 1. Schematic presentation of the immunoglobulins of normal human serum (adapted from Heremans¹).

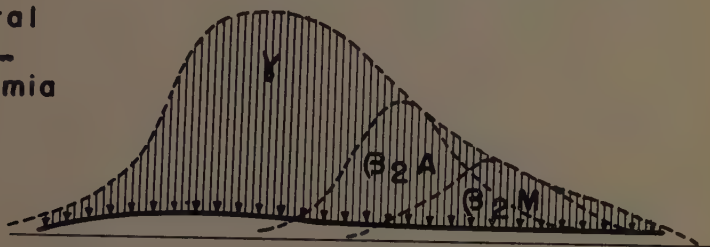
The cathodal end of an electrophoretic pattern is diagrammatically shown to be comprised of the three major antibody-containing fractions, γ , β_2A , and β_2M .

their electrophoretic mobilities that physicochemical differences do, indeed, exist.

The clonal selection theory of antibody production, formulated by Burnet,³ suggests that individual immunoglobulins are synthesized by specific clones of antibody-forming plasmacytes. Accordingly the diffuse decrease in the circulating immunoglobulins observed in the congenital and acquired hypogammaglobulinemic states, and the diffuse increase in these constituents observed in chronic infections, cirrhosis, sarcoidosis, the so-called collagen disorders and others appear to represent the result of pathological involvement of a large number, if not all, of the antibody-forming plasma cell clones. This postulate

is schematically represented in FIGURE 2. In contrast, the appearance of sharp, electrophoretically homogeneous peaks in the serum of patients with plasma cell myeloma and primary macroglobulinemia strongly suggests that these disease states and their accompanying protein abnormalities represent the effect of a malignant transformation of a single clone of plasma cells (or "plasmacytoid lymphocytes" in the case of primary macroglobulinemia) capable of synthesizing only one specific immunoglobulin component. At present, this

Congenital agammaglobulinemia



Diffuse hypergamma-globulinemias

Chronic infections
Rheumatoid arthritis
Systemic lupus erythematosus
Laennec cirrhosis
Boeck's sarcoidosis

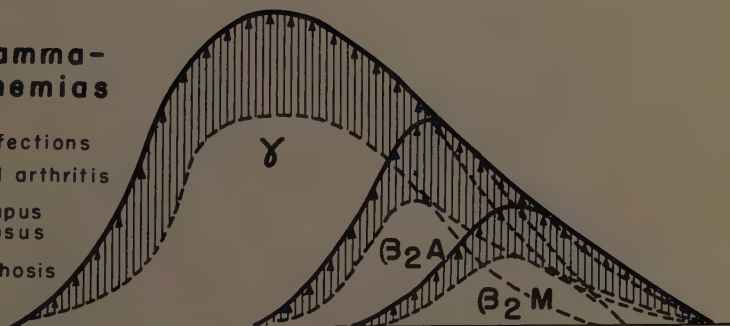


FIGURE 2. Schematic representation of immunoglobulins in congenital agammaglobulinemia and in the disease states associated with a diffuse increase in the gamma globulin fractions.

hypothesis is the one most consistent with the available physicochemical and immunochemical data. As diagrammatically shown in FIGURES 3 and 4, these data indicate that the myeloma serum proteins in individual cases may arise from either the major gamma fraction (FIGURE 3a) or the carbohydrate-rich, beta 2A fraction (FIGURE 3b), whereas the macroglobulins in primary macroglobulinemia are derived exclusively from the beta 2M fraction (FIGURE 4). The electrophoretic mobility of individual myeloma serum proteins or macroglobulins may vary over a wide range (indicated by the arrows in FIGURES 3 and 4) corresponding to the wide range of mobilities of their normal counterparts. To date, it is not known whether each of the myeloma globulins and

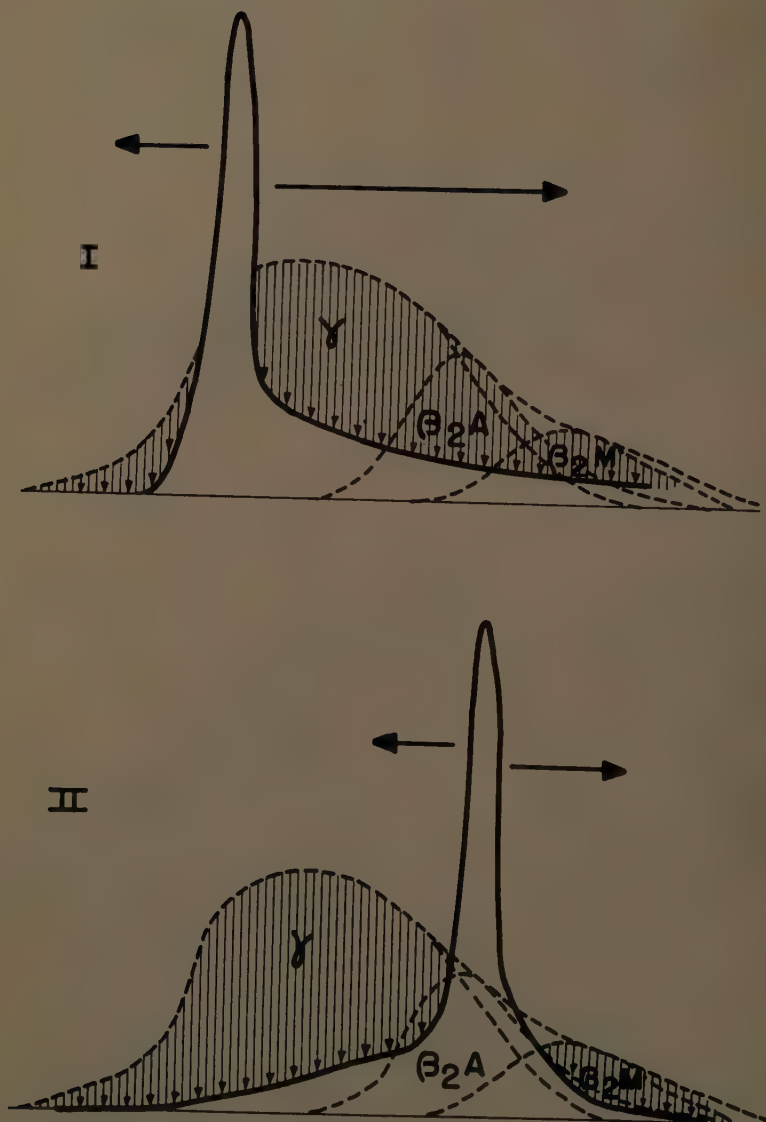
Plasma cell myeloma

FIGURE 3. Schematic representation of the two immunological types of myeloma serum globulins: (a) myeloma globulin immunologically related to the major gamma fraction; (b) myeloma globulin immunologically related to beta 2A. The horizontal arrows indicate the range of mobilities in individual cases. The concomitant decrease in the normal immunoglobulin constituents is also indicated.

each of the "pathological" macroglobulins has its exact counterpart in normal serum, that is, whether these paraproteins are normal constituents present in great excess or whether they are truly "abnormal." The failure to resolve this important issue is due to the limitations of presently available analytical techniques and may specifically be ascribed to our current inability to isolate *individual normal* immunoglobulins in sufficient quantity and native status to permit their characterization as individual proteins. Thus, with present methods, we are forced to compare all of the precise physicochemical and immunochemical data on *isolated* myeloma proteins and macroglobulins with

Primary macroglobulinemia

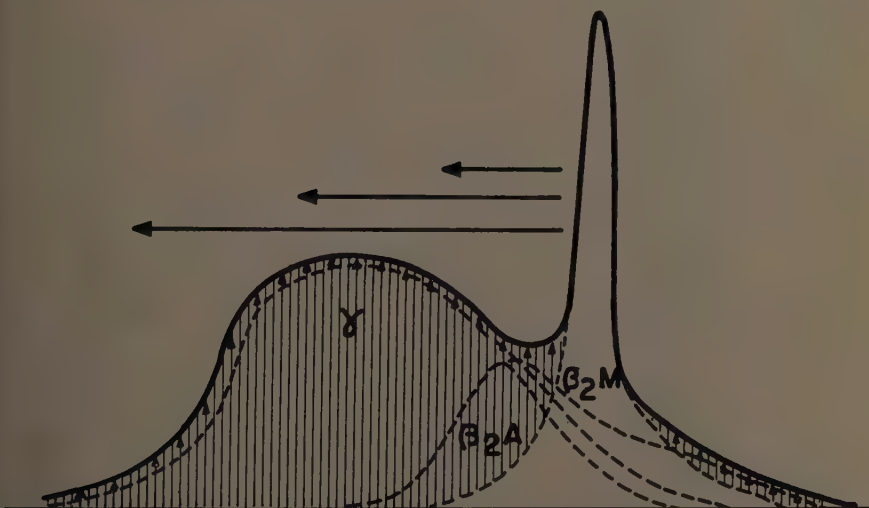


FIGURE 4. Schematic representation of the immunological relationship of macroglobulins in primary macroglobulinemia to the beta 2M fraction. In contrast to myeloma, the normal immunoglobulins in macroglobulinemia are usually not diminished.

corresponding data on *families* of normal proteins, for examples, Cohn Fraction II, chromatographic fractions of partially purified 19S macroglobulin, and fractions obtained by preparative ultracentrifugation. Whereas immunoelectrophoresis has not provided a full solution of this problem, the technique has at least permitted the delineation of the three major groups of immunoglobulins and the establishment of the relationship of the myeloma globulins and macroglobulins to these protein families.

Immunochemical Studies Prior to Immuno-electrophoresis

Prior to the development of immunoelectrophoresis, numerous studies of myeloma serum and urinary proteins were carried out, employing quantitative

precipitin, Ouchterlony and Oudin techniques (Hektoen and Welker,⁴ Moore *et al.*,⁵ Wuhrmann *et al.*,⁶ Kunkel *et al.*,⁷ Lohss and his co-workers,^{8,9} Deutsch and his co-workers,^{10,11} Slater *et al.*,¹² Smith *et al.*,¹³ and Korngold and Lipari¹⁴). These studies clearly established that the myeloma serum proteins and the urinary Bence-Jones proteins shared antigenic determinants in common with the normal gamma globulins. Comparable immunochemical studies of "pathological" macroglobulins (Habich and Hässig,¹⁵ MacFarlane *et al.*,¹⁶ Kanzow *et al.*,¹⁷ and Deutsch *et al.*¹¹) demonstrated that these paraproteins likewise cross-reacted with constituents present in normal serum, specifically with both 7S gamma globulin and 19S gamma macroglobulin fractions.

Immunoelectrophoretic Studies

Shortly after the development of the technique of immunoelectrophoresis by Grabar and Williams,¹⁸ the method was applied to the study of myeloma serum and urinary proteins (Grabar *et al.*,¹⁹ Burtin *et al.*,²⁰ Scheidegger,²¹ Martin and Scheidegger,²² Wunderly,²³ Scheiffarth *et al.*,²⁴ and Heremans^{1,2}) and to the macroglobulins (Burtin *et al.*,²⁵ Hartmann *et al.*,²⁶ Cleve and Schwick,²⁷ Sehon *et al.*,²⁸ Scheidegger *et al.*,²⁹ and Heremans¹). Similar immunoelectrophoretic studies have been in progress in this laboratory since 1958, and have been previously presented in only preliminary form (Osserman^{30,31}). Since the investigations in this field have been published almost exclusively in the European literature, the present report was deemed to be of possible value.

Materials and Methods

To date, immunoelectrophoretic analyses have been performed on the serum and urinary proteins from 140 cases of plasma cell myeloma and 20 cases of primary macroglobulinemia. Whereas the majority of these cases were under care at either the Francis Delafield Hospital or the Presbyterian Hospital, a small number of samples was submitted for study by physicians in other institutions, and we gratefully acknowledge this assistance.

The immunoelectrophoretic procedure was essentially that described by Grabar and Williams,¹⁸ employing photographic glass plates $3\frac{1}{4} \times 4$ inches or 5×7 inches overlaid with Veronal-Na Veronal buffer, pH 8.6, ionic strength 0.025, in a 1 per cent Difco "Noble" agar gel. Horse antihuman serum antiserum No. 491, obtained from the Pasteur Institute, Paris, and rabbit antigamma globulin antiserum (anti-Cohn Fraction II) were utilized for the immune development. The patterns were photographed prior to staining by employing the immunoelectrophoretic plates as negatives in a condenser-type enlarger. With this technique, virtually unlimited enlargement is feasible, providing greatly enhanced resolution.

Results

Normal serum and diffuse hypergammaglobulinemia. A normal serum pattern and the serum pattern of a patient with active rheumatoid arthritis are shown in FIGURE 5. These patterns and all subsequent ones, unless otherwise stipulated, were developed with the horse antihuman serum antiserum. The ovoid well in the beta-1 region is the antigen well. The cathodal displacement of

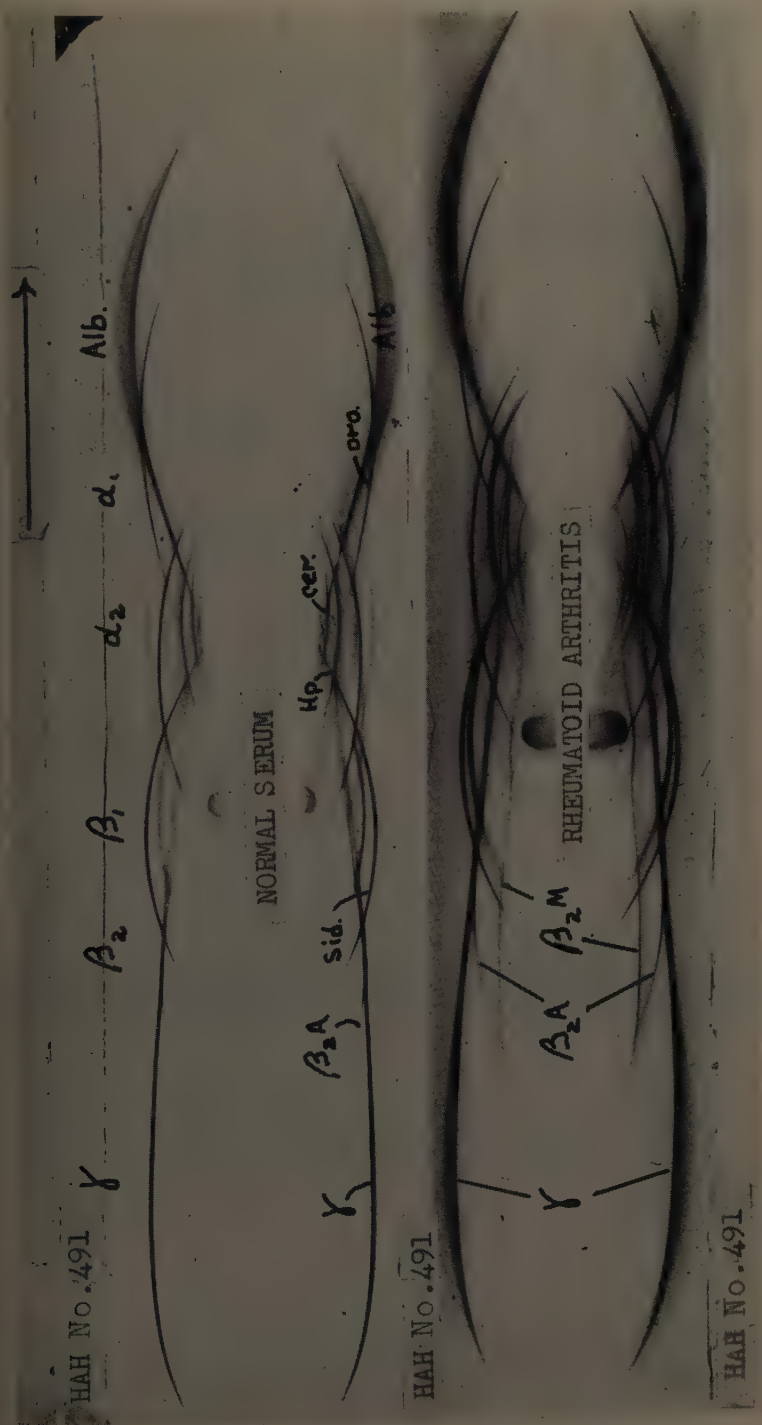


FIGURE 5. Immuno-electrophoretic pattern of normal serum (*above*) and of serum of a patient with active rheumatoid arthritis (*below*). The direction of electrophoretic migration is from left to right, as indicated by the arrow. HAH No. 491 = horse antihuman serum antiserum (Pasteur Institute No. 491). Alb. = albumin; oro = orosomucoid; cer = ceruloplasmin; Hp = haptoglobin; Sid = siderophilin. The three major components of the immunoglobulin system, gamma, beta 2A, and beta 2M, are better visualized in the rheumatoid serum pattern than in the normal serum.

the beta and gamma constituents is the result of electroendosmotic flow. It is evident that the gamma, beta 2A, and beta 2M arcs in the rheumatoid arthritis serum are diffusely and symmetrically increased as compared with the normal serum. The beta 2M arc of the normal serum cannot be visualized in this reproduction, and the beta 2A arc is seen only faintly.



FIGURE 6. Immuno-electrophoretic patterns of three gamma-type myeloma sera. The arc of the myeloma globulin in each pattern is indicated by an arrow: (a) the myeloma globulin occupies the cathodal end of the major gamma arc; (b) myeloma globulin in the mid-gamma region; (c) myeloma globulin in the anodal region of the major gamma arc. The complete fusion of the anodal and cathodal extremities of the respective myeloma proteins with the major gamma arc is evident.

Gamma-type myeloma globulins. Gamma-type myeloma globulins were found in 70 per cent of the sera displaying a characteristically homogeneous peak on electrophoretic analysis. Three representative patterns are shown in FIGURE 6. As seen in this figure, the immuno-electrophoretic appearance of the γ -type myeloma protein is that of a localized thickening of a restricted region of the long gamma arc with complete fusion of the anodal and cathodal

ends of the arc of the paraprotein with the gamma arc. In individual cases, the myeloma globulin may occupy any position along the extent of the major gamma arc from its extreme cathodal end to the anodal end, that is, into the beta mobility region. A concomitant diminution in the normal portions of the gamma arc, as well as a decrease in beta 2A and beta 2M, is also observed.

Beta 2A myeloma globulins. Thirty per cent of the myeloma globulins studied were found to be related immunologically to the beta 2A fraction. Three representative β_2A myeloma patterns are shown in FIGURE 7. In 7A, the paraprotein occupies a position at the cathodal end of the B_2A arc; in 7B, it is in the mid- β_2A region; and in 7C, it is at the anodal end of the β_2A arc, that is, in the alpha 2 mobility region. The myeloma protein shown in 7C corresponds to what has been termed an "alpha myeloma."

To date, it has not been possible to detect any clinical or pathological differences between the gamma and the beta 2A-type myeloma cases, but further studies in this area are in progress.

Evolution of a gamma-type myeloma protein with disease progression. Two serum patterns from the same patient obtained over a 10-month interval are shown in FIGURE 8. The initial pattern demonstrates, as the only detectable abnormality, a minimal distortion of the major gamma arc in the anodal region. With progression of disease, this abnormality became more evident, as illustrated in the lower pattern in FIGURE 8. It is noteworthy that, whereas the arc of the paraprotein fused completely with the gamma arc, the gamma arc itself was only partially interrupted. This behavior suggests that the myeloma protein was deficient in certain antigenic determinants present in the major gamma fraction.

A diminution of the beta 2A arc in this 10-month period of disease progression is also seen in FIGURE 8.

Bence-Jones urinary proteins. The low molecular weight (approx. 40,000) Bence-Jones type proteins excreted in the urine in 50 to 60 per cent of myeloma patients have been demonstrated to be related immunologically both to the gamma and to the beta 2A fractions. Although these proteins are present in the serum of these patients, the rapid clearance rate results in serum concentrations that are too low to be detectable by conventional electrophoretic techniques. As shown schematically in FIGURE 9, free electrophoresis or zone electrophoresis of the serum in patients whose neoplastic plasma cells are producing only a Bence-Jones type protein usually shows, as the only abnormality, a decrease in the gamma globulin, whereas the urine electrophoretic pattern will demonstrate a characteristically homogeneous protein.

The presence of the Bence-Jones protein in the serum, that is, Bence-Jones proteinemia, however, can be demonstrated by immuno-electrophoretic analysis. This is illustrated in FIGURE 10, and confirmed by the procedures of: (1) adding an aliquot of the urinary protein to normal serum, reproducing the abnormality in the patient's serum; and (2) adding the patient's urinary protein to his own serum, which resulted in an accentuation of the pathological arc in the fast gamma region. The characteristic immuno-electrophoretic appearance of the Bence-Jones protein in the serum is that of a "blister" on the major gamma arc, rather than a distortion of the full thickness of this arc (or of the beta 2A arc), which was observed with the myeloma serum globulins. Although it is not

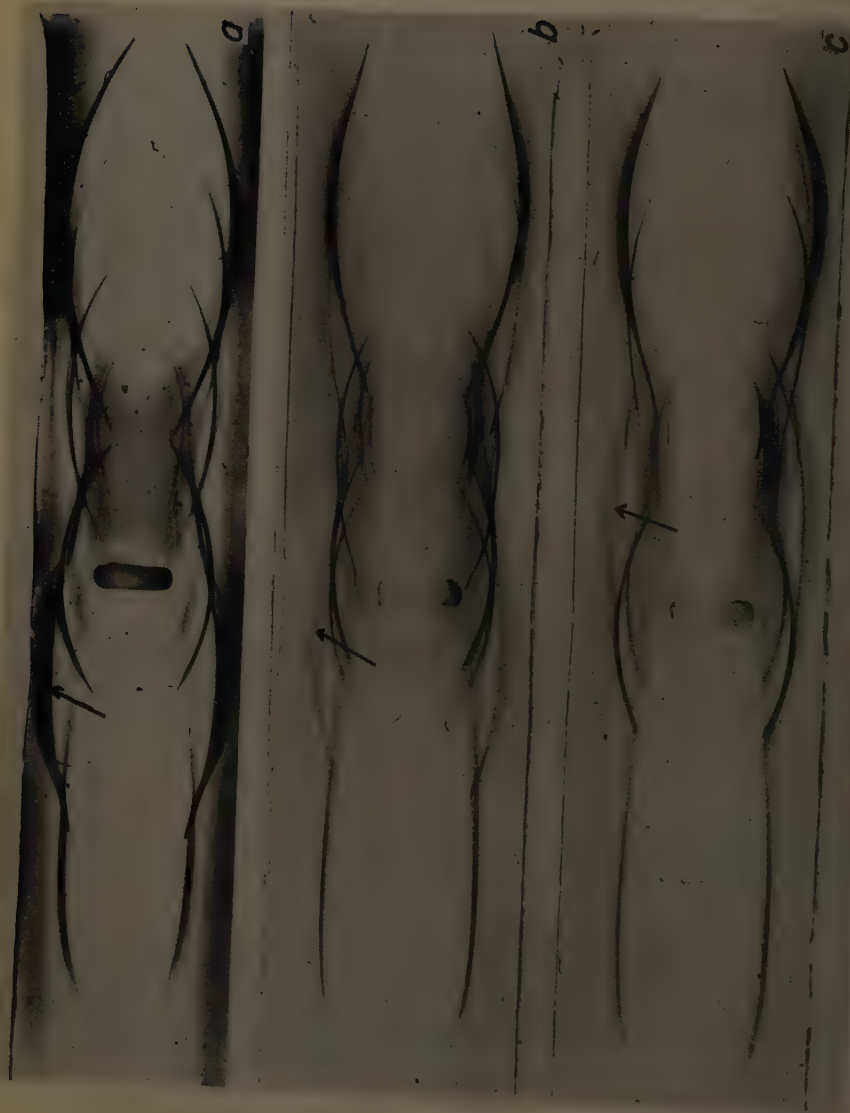


FIGURE 7. Immunoelectrophoretic patterns of three beta 2A-type myeloma sera. The arc of the myeloma globulin in each pattern is indicated by an arrow: (a) the myeloma globulin occupies the cathodal end of the beta 2A arc; (b) myeloma globulin in the mid-beta 2A region is indicated by an arrow; (c) myeloma globulin occupies the anodal end of the beta 2A arc. In contrast to the gamma-type myeloma globulins illustrated in Figure 6, the myeloma globulin in the mid-beta 2A region is indicated by an arrow.

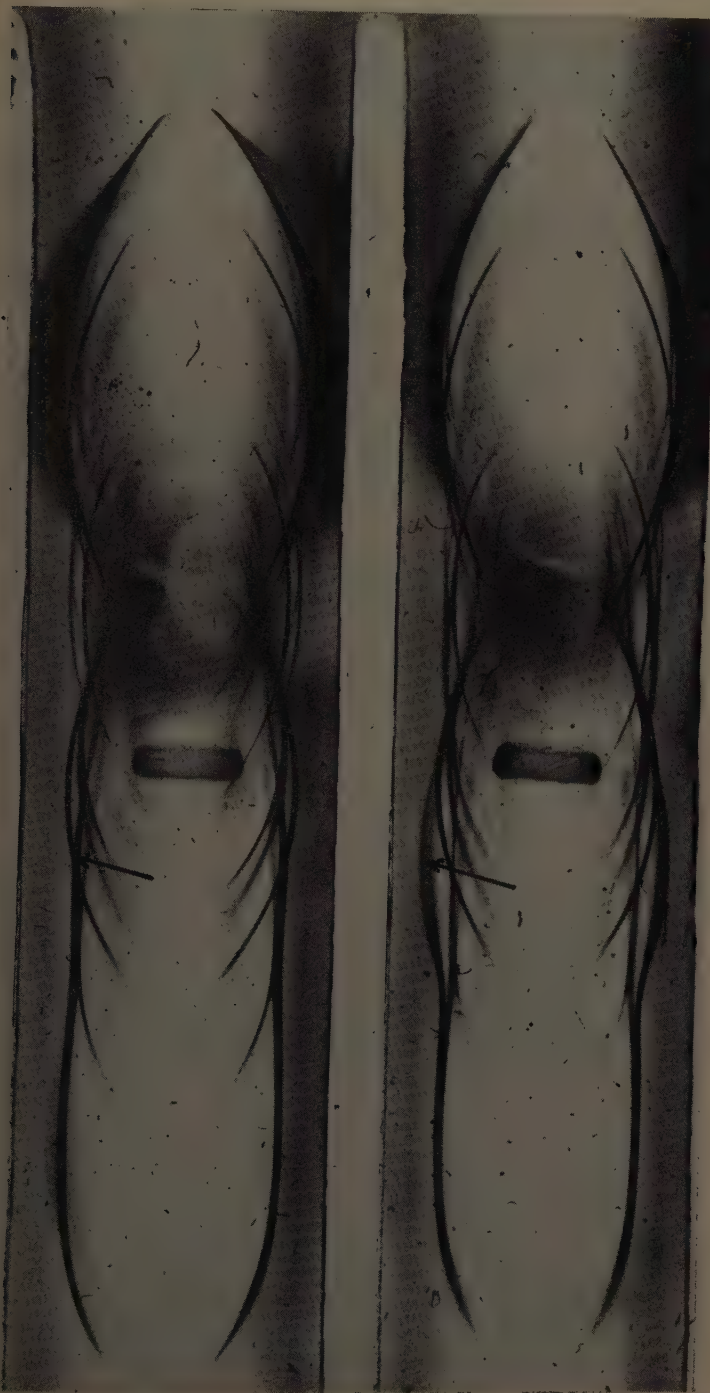


FIGURE 8. Sequential immunoelectrophoretic patterns of serum of a gamma-type myeloma patient over a 10-month interval during which there was disease progression. In the initial (*upper*) pattern, the only abnormality is a slight distortion of the anodal end of the gamma arc (*arrow*). Ten months later (*lower pattern*) this abnormality is clearly evident.

clearly seen in FIGURE 10, the anodal end of the Bence-Jones arc fuses with the beta 2A arc, indicating that these Bence-Jones proteins share immunological determinants with both the gamma and the beta 2A fractions.

Identification of the urinary proteins in cases with complex proteinuria. In many patients with myeloma, renal functional impairment develops in the course of disease progression, either due to the pathological consequence of

Plasma cell myeloma

III

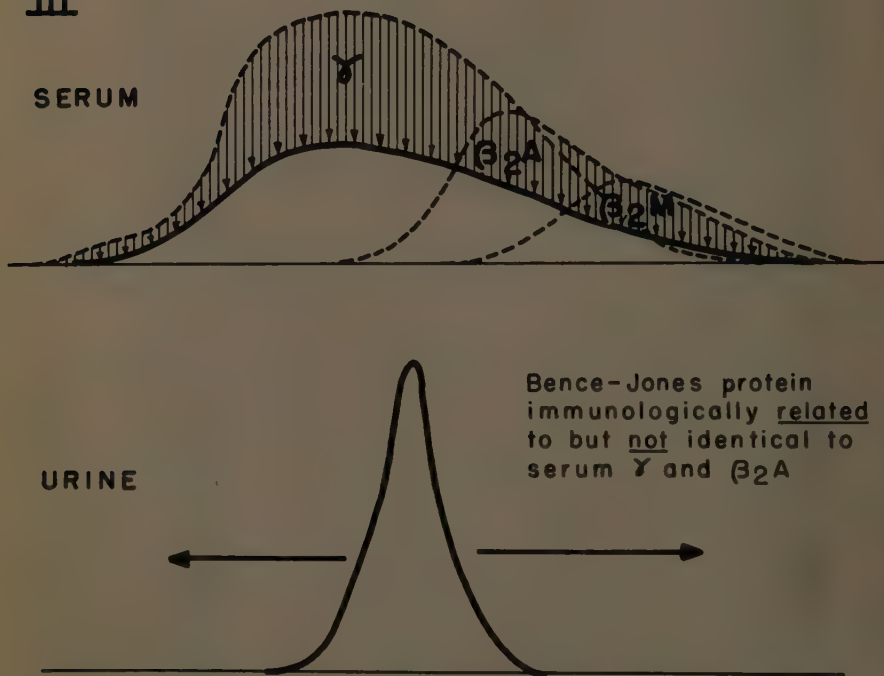


FIGURE 9. Schematic representation of the immunological relationships of the Bence-Jones proteins. The absence of a detectable serum peak on conventional electrophoretic analysis and the concomitant decrease in all three immunoglobulin fractions of this serum are also indicated.

Bence-Jones proteinuria or, in the absence of Bence-Jones proteinuria, as the result of such factors as hypercalcemia, infection, and anemia. In the latter patients, the urine may contain many of the serum protein constituents, as illustrated in FIGURE 11. As seen in this figure, the patient's serum showed a gamma-type paraprotein. There was no Bence-Jones protein in the urine, but there were at least three other major protein constituents, namely, albumin, a gamma globulin corresponding in mobility to the myeloma globulin, and an unidentified beta constituent. By a simple modification of the immunoelectrophoretic procedure (Osserman³¹), it is possible to identify the urinary pro-

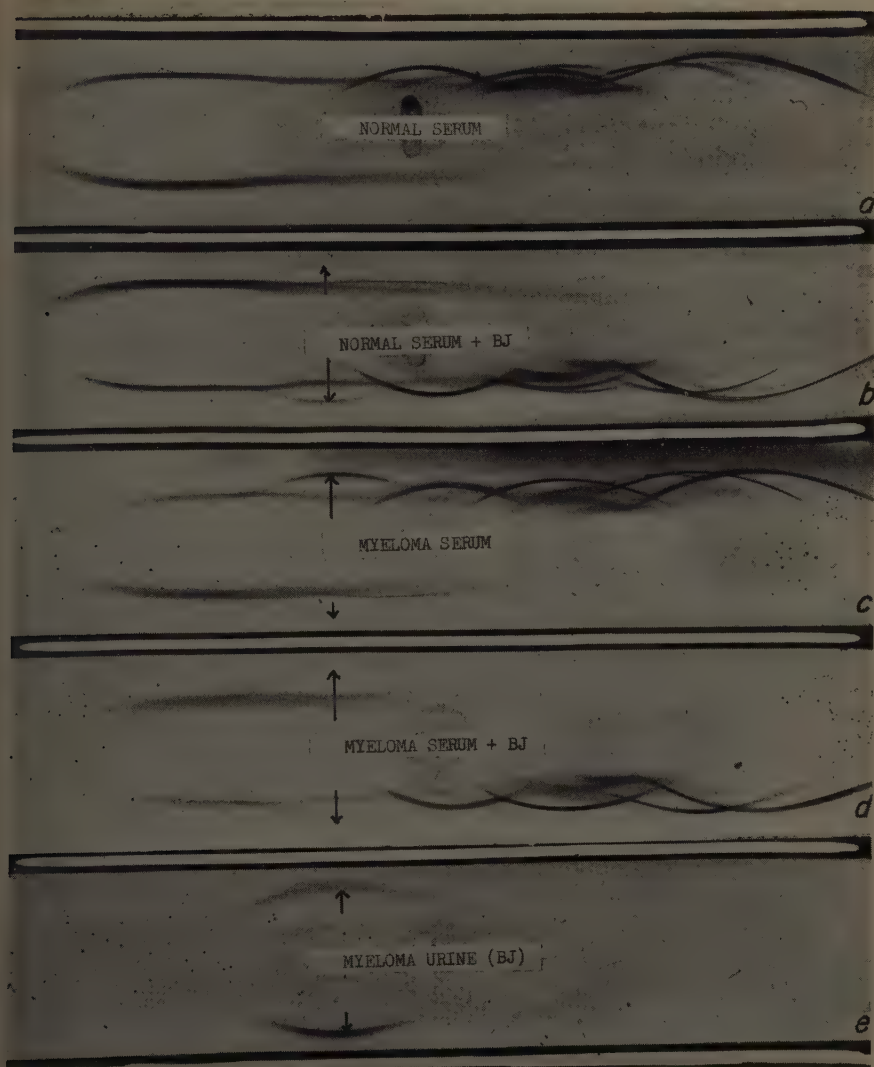


FIGURE 10. Immuno-electrophoretic demonstration of Bence-Jones proteinemia. The horse antinormal serum antiserum (HAH No. 491) and the rabbit antigamma (Fraction II) antiserum (RAG) were placed in alternate troughs. The Bence-Jones arc is indicated by arrows in each pattern. (a) Normal serum; (b) normal serum + Bence-Jones protein; (c) patient's serum; (d) patient's serum + Bence-Jones protein (the pathological arc has been displaced toward the antiserum trough and partially solubilized by antigen excess); (e) Bence-Jones protein. This is the patient's whole urine, concentrated by osmodialysis. The absence of albumin and of any serum constituents other than the Bence-Jones protein is evident.

teins conclusively. As shown in the lower section of FIGURE 11, a trough is cut in the agar following the electrophoretic separation of the serum and a concentrated solution of the urinary proteins is placed in this trough. The urine proteins diffuse from the trough along a straight diffusion front toward the antiserum, producing straight lines of immune precipitation, interrupted

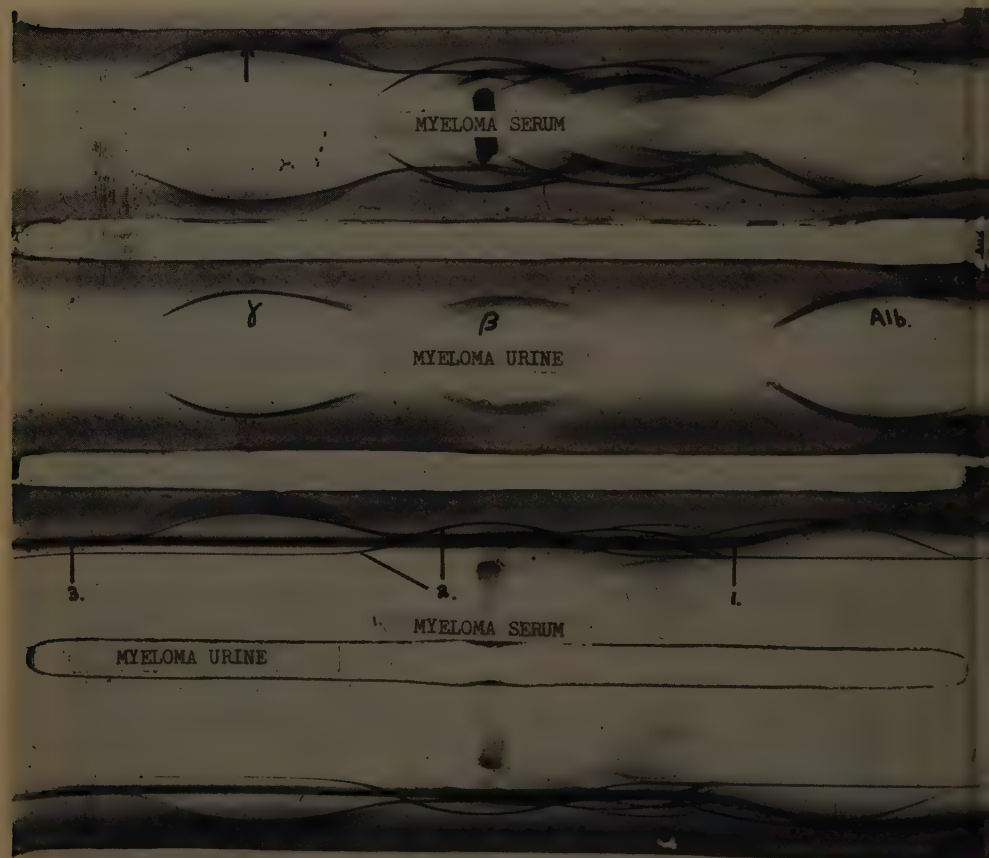


FIGURE 11. Identification of the urinary protein constituents in complex proteinuria without Bence-Jones proteinuria. (a) Patient's serum, demonstrating a gamma-type abnormality; (b) patient's urine: albumin, unidentified beta, and gamma constituents; (c) modified double diffusion system, confirming the identity of the albumin (1) and gamma (3) components, and establishing the beta constituent (2) as siderophilin.

only by the arcs of their corresponding proteins in the serum pattern. In the example shown in FIGURE 11, it was possible by this means to identify the urinary beta constituent as siderophilin.

Primary macroglobulinemia. FIGURE 12 demonstrates the characteristic immunoelectrophoretic pattern of the serum in Waldenström's macroglobulinemia. The arc produced by the paraprotein is seen to occupy a position inside the gamma and beta 2A arcs, reflecting the larger molecular size and slower



FIGURE 12. Immuno-electrophoretic pattern of the serum in primary (Waldenström's) macroglobulinemia. A normal serum pattern (*above*) is shown for comparison. The paraprotein (*arrow 1*) occupies the cathodal end of the beta 2M arc. The long extension into the beta region (*arrow 2*) of the beta 2M arc may in part be due to "trailing."

diffusion rate of the macroglobulin. Whereas the elongation of this beta 2M arc may, in part, reflect a "trailing" effect in the agar medium, it may also be due to a concomitant increase in several other constituents of this immunoglobulin fraction. As mentioned previously, it is noteworthy that the gamma and beta 2A arcs in macroglobulinemia are frequently not attenuated in the manner observed in the myeloma sera.

Approximately one third of the macroglobulins fail to migrate through the agar gel, resulting in a crescent-shaped zone of spontaneous precipitation immediately adjacent to the antigen well. This type of pattern is suggestive but not diagnostic of macroglobulinemia. The type of pattern illustrated in FIGURE 12, however, is diagnostic of macroglobulinemia, and may be used in place of analytical ultracentrifugation for clinical purposes.

Summary

Immunoelectrophoretic analysis of the serum and urinary proteins in myeloma and macroglobulinemia represents an extremely sensitive and accurate technique for the detection and characterization of these constituents. The immunoelectrophoretic approach permits the classification of the myeloma serum globulins into two major immunological groups, namely, those related to the major gamma fraction, and those related to beta 2A. The low molecular weight Bence-Jones urinary proteins can be shown to possess immunological determinants of both gamma and beta 2A. The sensitivity of the technique also makes possible the demonstration of minute quantities of Bence-Jones protein in the serum of certain myeloma patients. This phenomenon has been confirmed (1) by the addition of urinary Bence-Jones protein to the corresponding patient's serum and demonstration of an enhancement of the serum Bence-Jones precipitin arc, and (2) by the addition of Bence-Jones protein to normal serum, with reproduction of the pattern observed in the myeloma patient's serum.

In cases of plasma cell myeloma with complex proteinuria, consisting of several different serum protein constituents, a three-reactant modification of the immunoelectrophoretic technique has permitted the precise identification of the several urinary proteins. Of particular interest has been the demonstration of significant quantities of siderophilin in the urine of certain myeloma patients.

The serum immunoelectrophoretic pattern in primary macroglobulinemia has been demonstrated to be specific and diagnostic. These high molecular weight paraproteins are immunologically primarily related to the beta 2M fraction. Serum immunoelectrophoresis has been established to be a valid substitute for ultracentrifugal analysis in the diagnosis of Waldenström's macroglobulinemia.

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ABNORMAL PLASMA COMPONENTS AND THEIR SIGNIFICANCE IN DISEASE*

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The term "abnormal serum proteins" may be defined in several ways: quantitatively, qualitatively, and functionally. Sometimes it may be difficult to distinguish between these three definitions. For example, a rabbit immunized with an antigen may become hypergammaglobulinemic; its γ globulins will contain antibodies lacking from the serums of other rabbits, that is, they can combine with the antigen used for immunization, and this functional capacity must involve certain configurational groupings on the antibody molecule lacking from normal γ globulin (however, it should be mentioned that some theoreticians postulate that the serum of every individual contains "antibodies" against the substances that later may induce immunity¹).

Abnormalities have been observed for many serum proteins, but the present discussion will deal with the globulins that Heremans designates as "the immunoglobulins."² These immunoglobulins are very heterogeneous: their electrophoretic mobilities may range from the α_2 -globulin region to that of the very slow γ_2 globulins; their molecular weight varies between 150,000 to 1,000,000 or more, and they comprise several antigenic species.³ Functionally, however, they have a common denominator: their ability to become antibodies.²

In so far as the nomenclature of these globulins is concerned, we shall follow the terminology commonly used by most workers in this country: γ_2 globulin refers to those molecules with the slowest electrophoretic mobilities, and contains molecules with a molecular weight of 150,000 (7S). By immunoelectrophoresis it may be shown that antigenically this protein(s) extends into the fast β globulin region.

γ_1 Globulin refers to the valley between the γ_2 -globulin peak and the β globulin peak. γ_1 Globulin contains three molecular species:

(1) a protein antigenically indistinguishable from γ_2 globulin, mol. wt. 160,000.

(2) γ_1A (β_2A globulin). This protein, first noted by immunoelectrophoretic studies, was recently isolated and characterized by Heremans *et al.*,⁴ an important achievement. It is heterogeneous in the ultracentrifuge, but the main component has a sedimentation constant of 7S. Antigenically, it is related to but not identical with, γ_2 globulin.

(3) The γ_1 macroglobulin (β_2M). This protein comprises approximately 30 per cent of the γ_1 globulins and has a molecular weight of 1,000,000 (19S). It is antigenically related to γ_2 globulin.⁵

Quantitative abnormalities may affect all these proteins, individually or jointly. For example, congenital agammaglobulinemia is characterized by

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the low levels of all immunoglobulins,⁶ whereas various other diseases (lupus erythematosus, kalaazar, cirrhosis, and others) frequently lead to a diffuse hypergammaglobulinemia in which all components may be elevated.

Marked increases of only one component, with frequent decreases of the others, are most often encountered in multiple myeloma and macroglobulinemia of Waldenström, and it is with these two diseases that most of this paper will be concerned.

MATERIALS AND METHODS

Electrophoresis

Moving boundary electrophoresis. Moving boundary electrophoresis was performed in the standard cell (11 ml.) of the Spinco model H electrophoresis-diffusion instrument. Veronal buffer, $pH\ 8.6\ \mu = 0.1$, was used throughout; each run was of 180 min. duration at 16 mAmp.

Paper electrophoresis. All serums studied were analyzed by paper electrophoresis with the Spinco model R instrument. Veronal buffer, $pH\ 8.6\ \mu = 0.075$, was used.

Zone electrophoresis on starch. This process was performed, as described previously,⁷ with phosphate buffer, $pH\ 7.6\ \mu = 0.1$.

Immunoelectrophoresis. Microimmunoelectrophoresis was performed according to Scheidegger⁸ with several modifications that will be described elsewhere. Phosphate buffer, $pH\ 7.6\ \mu = 0.05$, was used.

Ultracentrifugal Analysis

Ultracentrifugal analysis was performed in the Spinco model E analytical ultracentrifuge at 59,780 rpm.

Immunological Methods

Antigens. Normal γ globulins, purified myeloma proteins, and macroglobulins were prepared by zone electrophoresis on starch.⁷ γ_1 -A (β_2 A) globulin was prepared according to Heremans *et al.*⁴ or according to our own methods (unpublished data). Leukocyte antigens were prepared as described elsewhere.⁹

Antisera. Rabbits were immunized with leukocyte extracts,⁹ purified γ globulins, myeloma proteins, or macroglobulins. All antigens were incorporated in Freund adjuvant. After the resulting antisera were found to have the desired properties, as determined by trial bleedings and subsequent immunological tests, the rabbits were exsanguinated. The absorptions of the antisera with purified proteins or protein fractions in order to increase their specificity has been described.^{10,11}

Immunological Tests

The double diffusion test of Ouchterlony¹² was used throughout the immunological work, as was the microimmunoelectrophoresis method described above.

RESULTS*

The immunoglobulins. Many potent antisera against γ globulin react with all three immunoglobulins. FIGURE 1 illustrates the reactivity of such an antiserum. This figure also illustrates the many variations that may be seen



FIGURE 1. Immunoelectrophoresis of normal human serum and serums of seven chronic lymphocytic leukemics. See text for explanation.

* The following abbreviations are used: MM, multiple myeloma; MG, macroglobulin; and MGW, macroglobulinemia of Waldenström.

when sera* from patients with chronic lymphocytic leukemia are examined by immunoelectrophoresis. The first serum is representative of the patterns obtained when normal sera are examined. The three immunoglobulins are clearly visible: γ_2 globulin forms the line that extends farthest to the negative electrode; γ_1A globulin is the curved line that ends approximately halfway between the origin and the end of the γ_2 -globulin line. The macroglobulin (β_2M) is the faint line that extends almost straight from the origin to the point where it meets the γ_1A -globulin line.

The second serum was from a patient with severe hypogammaglobulinemia. Only the γ_2 -globulin line is visible, and its position, far from the antibody trenches, indicates that there is very little of this antigen in the patient's serum. The third serum contains slightly more γ_2 globulin, but otherwise it is similar to serum 2. The fourth serum has approximately normal levels of γ_2 globulin and γ_1A globulin, but not enough macroglobulin to produce a visible precipitate. The fifth serum is characterized by a decrease in the γ_2 globulin and γ_1A globulin (the latter did not form a precipitate), but the macroglobulin is elevated. The sixth serum is approximately normal, and the last serum represents hyper γ_2 globulinemia and macroglobulinemia. No γ_1A was detectable. This serum is indistinguishable from that of patients with macroglobulinemia of Waldenström (see below), and part of the macroglobulin was a cryoglobulin. It should be emphasized that when either the γ_1A globulin or the macroglobulin becomes elevated it is difficult to determine which line is formed by which antigen and, in this study, special methods had to be employed to ascertain that the lines of sera 4 and 5 were indeed formed by γ_1A globulin and macroglobulin.

Multiple myeloma. Multiple myeloma, the clinical features of which have been reviewed by Snapper,¹³ is a malignancy of the plasma cell. Its two main biochemical characteristics are the presence of abnormal proteins in the sera of the patients and in the urines of 50 per cent of them. The abnormal serum proteins are best visualized by electrophoretic analysis; they appear as sharp peaks indicating a high degree of electrophoretic homogeneity. This contrasts with serums of patients with diseases that may give rise to an electrophoretically diffuse hypergammaglobulinemia. The γ_1A -myeloma proteins, however, are occasionally heterogeneous. FIGURES 2 and 3 illustrate these differences in the electrophoretic patterns. FIGURE 2 (top) is representative of a normal serum and FIGURE 2 (bottom) of a diffuse hypergamma globulinemic serum with marked elevations in both the γ_2 - and γ_1 -globulin regions. FIGURE 3 (top) shows the typical sharp peak seen with most MM serums and FIGURE 3 (bottom) illustrates the rather rare type of β_2A MM serum with a split peak. FIGURE 4 shows the characteristic immunoelectrophoretic patterns obtained with sera from patients with multiple myeloma. The electrophoretic mobilities of the MM globulins vary with each patient, and in our laboratory we have studied such proteins with mobilities ranging from the slow γ_2 -globulin region to the α_2 -globulin regions. An extensive list of references to papers dealing with immunoelectrophoretic analysis of MM sera and MG sera is given by J. Heremans.¹⁴

* Supplied by Daniel G. Miller of the Memorial Center for Cancer and Allied Diseases, New York, N.Y.

Electrophoretic analysis tells us relatively little about the abnormal nature of the MM globulins, since normal γ globulins have a similar electrophoretic distribution. It may be suggested therefore that the MM proteins are the elevated components of normal serum globulins.¹⁵ In order to study other abnormal features of the MM proteins it is necessary to apply immunological methods.

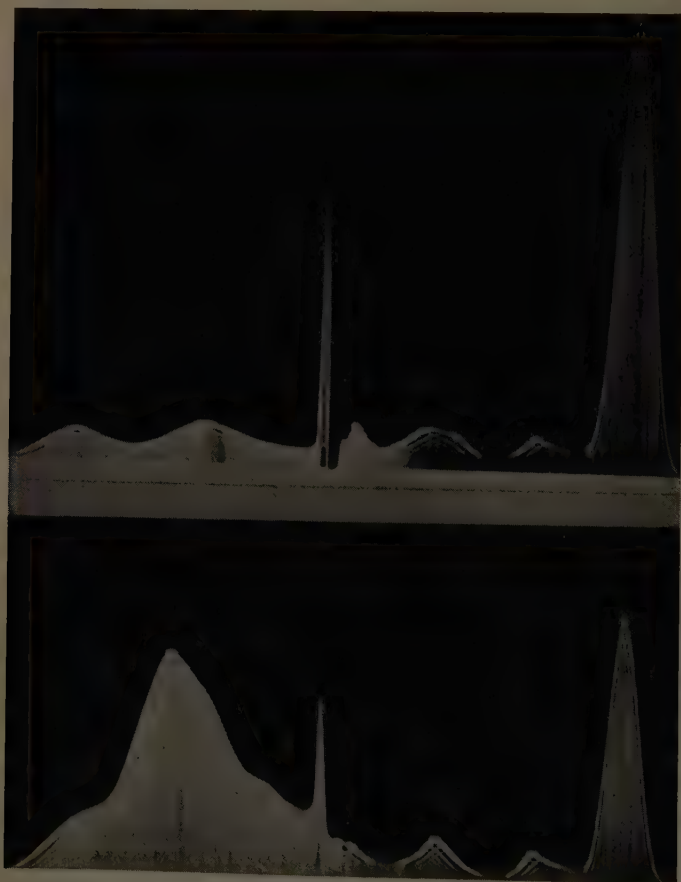


FIGURE 2. Moving boundary electrophoresis of normal serum (*top*) and hypergamma-globulinemic serum (*bottom*). Descending limb.

When rabbits (or other suitable animals) are immunized with normal γ_2 globulin the resulting antisera will contain antibodies capable of reacting with members of the immunoglobulins.^{7,14,16-19} When such antisera react with normal γ_2 globulins, a single zone of precipitate is formed, regardless of the electrophoretic mobility or the method of preparation of the γ_2 globulin. When different preparations of such γ_2 globulins are placed in adjacent cups the lines will coalesce completely. When MM globulins of the γ_2 -globulin family are tested in this manner, the lines coalesce only partially (FIGURE 5). This

partial coalescence and the resulting spur is characteristic of cross-reactions between closely related but not identical systems.¹² Double spurs (FIGURE 5) are formed when MM globulins belonging to different antigenic groups are compared,²⁰ suggesting that each lacks some antigenic determinants present in the other, and that all lack some antigenic determinants of normal γ_2 globulin. Although under these test conditions purified MM globulins usually



FIGURE 3. Moving boundary electrophoresis of serums of a γ_2 (*top*) and a γ_1A (*bottom*) multiple myeloma. Descending limb.

produce single zones of precipitate, some may produce two zones, even when the immunological reaction occurs at equivalence (FIGURE 5), indicating that there are two antigenically different components in these preparations.

The formation of double spurs has been found to be useful for the classification of MM proteins.⁷ MM globulins of group 1 are antigenically most closely related to normal γ_2 globulin; those of group 2 are more deficient. A third group, antigenically even more deficient than the first two, was shown by Heremans¹⁴ to be identical with the β_2A MM globulins of the European litera-



FIGURE 4. Immunoelectrophoresis of normal serum (*top*) and serums from multiple myeloma patients: the antiserum used to develop the immunoelectrophoresis was specific for γ_2 and γ_1A globulins. The MM serums belonged to the groups indicated on the figure.



FIGURE 5. The cross-reactions of MM globulins with anti- γ_2 -globulin serum (center cup). Cups A and D: normal γ_2 globulin. The numbers at the other cups indicate the MM group.

ture and group 2 of Slater *et al.*¹⁹ Of the 209 MM globulins examined in our laboratory, 46.5 per cent belonged to group 1, 17.7 per cent to group 2, and 35.8 per cent to group 3.

The electrophoretic mobilities of the group 3 globulins may range from fast α_2 to γ_2 globulin (FIGURE 6). In the ultracentrifuge several components usually may be detected, the main components having sedimentation constants of 7S and 9S.²¹



FIGURE 6. Immunoelectrophoresis of normal serum (*top*) and eight sera from γ_1A -MM patients. An anti- γ_1A -MM globulin serum was used to develop the patterns. The faster moving component is the γ_1A globulin, the slower the γ_2 globulin.

γ_1A , (β_2A) globulin, first detected by immunoelectrophoresis,²² is electrophoretically heterogeneous. Its greatest concentration is in the β -globulin region and it extends from there into the γ_1 -globulin region. It has a sedimentation constant of 7S, and cross-reacts antigenically with γ_2 globulin, that is, it has antigenic determinants in common with this protein.¹⁴ FIGURE 7 shows the immunoelectrophoretic patterns of two preparations of γ_1A globulins prepared according to Heremans *et al.*⁴ and by our own procedures (L. Korngold, unpublished data). Since all these procedures include an electrophoretic separation, it is possible to obtain γ_1A globulins with differing mobilities (FIGURE 7). The γ_1A globulins are antigenically heterogeneous, being composed of at



FIGURE 7. The electrophoretic mobilities of two samples of purified γ_1 A globulin (center). Top and bottom: normal serum. The antiserum was prepared by inoculating a rabbit with a γ_1 A-MM globulin.
FIGURE 8. Antigenic relationships of γ_2 globulins, purified normal γ_1 A and γ_1 A-MM globulins.
Center anti- γ_2 -globulin serum; (A) γ_2 globulin; (B and E) purified normal γ_1 A globulin (traces of γ_2 globulin still are visible); (C, D, and F) three differ-

least two components (FIGURE 8), both of which cross-react with normal γ_2 globulin. Moreover, these two components of the γ_1A systems have their counterparts in the γ_1A MM globulins (FIGURE 8).

On the basis of the above data many investigators have suggested that the MM globulins are antigenically abnormal proteins. Other workers, however, maintained that normal serum contains similar proteins, but at very low concentrations.^{18,23} My associates and I therefore developed theoretical models dealing with the postulated antigenic heterogeneity of γ_2 globulins, based on the assumption that these proteins were antigenically closely related but not identical. It was postulated that the injection of normal γ_2 globulins into a rabbit would lead to the production of antibodies against those determinants all γ_2 globulins had in common, as well as against those specific for each sub-family of γ_2 globulins. The antiserum of such an animal would therefore produce only one line with normal γ globulin, since all antigen molecules would be incorporated in the same immune framework. This conclusion is based on the assumption that the various antigenic subfamilies of the γ_2 globulins are represented approximately in equal amounts (in multiple myeloma one of the subfamilies would be very elevated with the result that two zones of precipitate would be formed).

In order to produce antisera capable of distinguishing between the different components of normal γ_2 globulin it would therefore be necessary to immunize rabbits with γ globulins lacking most of the antigenic determinants of normal γ_2 globulin. We therefore immunized many rabbits with γ globulins from other mammalian species as well as with purified preparations of MM globulins and MGW globulins, all of which were known to be antigenically deficient with respect to normal γ_2 globulin. Many of the antisera so produced had the anticipated properties; that is, they formed at least two zones of precipitate with normal human γ_2 globulin. The reaction of two such antisera is shown in FIGURE 9. In this experiment the antisera were prepared by immunizing rabbits with MGW. Another example is presented in FIGURE 7, in which an antiserum against a γ_1A MM globulin was used. Here normal serum was used for immunoelectrophoresis, but similar results have been obtained with purified γ_2 globulin. An example of the formation of two lines with normal γ_2 globulin by double gel diffusion (Ouchterlony) is given in FIGURE 11e, cup E; in this instance the antiserum was against a group 2 MM globulin (Edelman *et al.*²⁴ have independently shown that such antisera can be prepared by immunizing rabbits with pathological human globulins; Dray²⁵ immunized rhesus monkeys with human γ globulin and obtained antisera capable of forming more than one line with human γ globulin). These findings seem to lend support to the assumption that MM globulins are elevated components of the normal γ globulins; for example, group 1 and group 2 globulins may be the elevated counterparts of the two families of γ_2 globulins that formed the two zones of precipitate illustrated in FIGURE 9, whereas the group 3 MM globulins are the elevated members of the γ_1A -globulin family. Preliminary data from our laboratory, however, suggest that this explanation is an oversimplification. FIGURE 10 shows the reaction of one antiserum capable of producing two lines with normal γ_2 globulins, with several MM globulins belonging either to group 1 or 2. It is apparent that these MM globulins produce one line only with this antiserum;



FIGURE 11. (a to i): The reactivity of anti-MM globulin sera with homologous and cross-reacting antigens. The group of the MM globulin is indicated by numerals in parentheses. All antigens at 2 mg./ml.

(a) Center cup: anti-Nu (1) serum. (A and D) Nu (1); (B) La (2); (C) Pa (1); (E) γ_2 globulin; (F) Cr (2).

(b) Center cup: anti-Ro (1) serum. (A and D) Ro (1); (B) γ_2 globulin; (C) MM (1); (E) Pa (1); (F) Ma (1).

(c) Center cup: anti-Pa (1) serum. (A and D) Pa (1); (B) La (2); (C) Nu (1); (E) γ_2 globulin; (F) Cr (2).

(d) Center cup: anti-Ko (2) serum. (A and D) Ko (2); (B) γ_2 globulin; (C) MM (1); (E) Pa (1); (F) Cr (2).

(e) Center cup: anti-Cr (2) serum. (A and D) Cr (2); (B) normal serum (undiluted); (C) La (2); (E) γ_2 globulin; (F) Pa (1).

(f) Center cup: anti-La (2) serum. (A and D) La (2); (B) γ_2 globulin; (C) Cr (2); (E) Nu (1); (F) Pa (1).

(g) Center cup: anti-We (3) serum. (A and D) We (3); (B) Ma (3); (C) γ_1 A globulin; (E) Wa (3); (F) Mc (3).

that all these lines fuse to give a "reaction of identity," and that these lines finally fuse with only one of the two lines produced by normal γ_2 globulin. From this result it might be concluded that the MM globulins of groups 1 and 2 are all related to the same component of the γ_2 globulins.

Moreover, many investigators have found that antisera against MM globulins contained antibody specific for the homologous antigen.^{7,17,19,26} In FIGURES 11a to i some of the results obtained with antisera against highly purified MM globulins are summarized. The results obtained with antisera against group 1 MM globulins are illustrated in FIGURES 11a to c. In all instances the homologous antigens formed spurs with γ_2 globulin and all other MM globulins tested. In most instances spurs were also formed between the lines of the heterologous MM globulins of groups 1 and 2 (FIGURES 11a and c), since the group 2 MM globulins are antigenically more deficient when tested with these antisera. With antisera against MM globulins of group 2 (FIGURES 11d, e, and f), spurs were formed between the homologous antigen and all other globulins tested, even when undiluted normal serum was used (FIGURE 11e, cup B). Note that when an antiserum forms two zones of precipitate with normal γ_2 globulin (FIGURE 11e, cup E) both lines coalesce partially with the line formed by the homologous antigen and only one of these lines coalesces with the single line formed by an MM globulin of group 1 (cup F). With most of these antisera, spurs were formed between the heterologous MM globulins of groups 2 and 1, the latter being antigenically more deficient in this instance. FIGURES 11g to i illustrate the reactions of antigroup 3 MM globulin sera with the homologous and cross-reacting MM globulins of group 3, and the cross-reacting normal γ_1 A globulin (similar results are obtained when whole normal serum is substituted for γ_1 A globulin, or with antisera absorbed with γ_2 globulin). Many of these antisera, besides distinguishing the homologous antigen from all other MM globulins and normal γ globulins, can distinguish between MM globulins of groups 1 and 2 and, at times, between subgroups of a given group.

In our experience every MM globulin contains unique antigenic determinants; consequently if we are to assume that MM globulins are the elevated components of normal γ globulin, we must conclude that there are as many different γ globulins in the serum of a normal individual as there are patients with multiple myeloma.

Macroglobulinemia

There are many diseases in which the γ_1 macroglobulin is elevated. Nevertheless, the term macroglobulinemia has become almost synonymous with

FIGURE 11—*Continued.*

(h) Center cup: anti-Wa (3) serum. (A and D) Wa (3); (B) Ma (3); (C) γ_1 A globulin; (E) We (3); (F) Mc (3).

(i) Center cup: anti-Mc (3) serum. (A and D) Mc (3); (B) Ma (3); (C) γ_1 A globulin; (E) Wa (3); (F) We (3).

(j to l): The reactivity of anti-MGW serums with homologous and cross-reacting MGW antigens. All antigens at 3 mg./ml.

(j) Center cup: anti-Lo serum. (A and D) Lo; (B) normal serum (undiluted); (C) Cr; (E) Tc; (F) Pu.

(k) Center cup: anti-Re serum. (A and D) Re; (B) Tc; (C) Pu; (E) Cr; (F) Lo.

(l) Center cup: anti-Pu serum. (A) Pu; (B) Re; (C) Tc; (D) Lo; (E) normal mg., purified; (F) normal serum (undiluted).

macroglobulinemia of Waldenström. For a long time this syndrome could be diagnosed only by ultracentrifugal analysis of the patient's serum; if the macroglobulin exceeded an arbitrarily chosen value of 5 per cent of the serum proteins,²⁷ the patient was considered to have macroglobulinemia of Waldenström. This test (and the same is true for the presently available immunological tests^{28,29}) cannot distinguish the early stages of this disease from other conditions in which the macroglobulin may become elevated.

Serums from patients with advanced MGW are indistinguishable by electrophoretic analysis from those of patients with multiple myeloma.³ FIGURE 12 is an example of such a MGW serum; the abnormal serum protein has a mobility of a slow γ_2 globulin. Most macroglobulins, normal and abnormal, have the mobility of γ_1 globulin, but proteins with mobilities of α_2 globulin or β globulin may be encountered.¹⁰ FIGURE 13 illustrates this variability among macroglobulins. The ultracentrifugal pattern of a MGW serum is illustrated in FIGURE 14. Antigenically, MGW differs from normal MG by its lack of



FIGURE 12. Moving boundary electrophoresis of a serum from a patient with macroglobulinemia of Waldenström. Descending limb.

antigenic determinants.¹⁰ This can be demonstrated with antisera against normal γ globulin. Since these determinants are also shared by the 7S γ_2 globulins¹¹ it has not been possible as yet to develop diagnostic tests based on this qualitative difference as has been done for MM.²⁹ Besides being antigenically deficient, MGW contain determinants lacking in normal MG and, like the unique MM determinants, they are specific for each patient.¹¹ Antisera against MGW absorbed with normal serum constituents cannot therefore be used diagnostically, since they will react with the homologous antigen only. The specificity of these antisera is indicated in FIGURES 11j to 11l.

Besides differences in electrophoretic mobility and antigenic specificity, macroglobulins from different patients differ widely in other biochemical characteristics. These differences have been recently reviewed by F. W. Putnam³ and by H. G. Kunkel.³⁰

Cryoglobulins

The term "cryoglobulin" refers to the property of certain proteins, usually from patients with multiple myeloma or macroglobulinemia, to precipitate at

low temperatures. The cryoglobulins that we have examined from patients with multiple myeloma belonged mostly to antigenic group 1 (see above); only one example of a group 2 cryoglobulin has been seen in our laboratory to date. The macroglobulins that are also cryoglobulins cannot be distinguished immunologically from those that are not cryoglobulins; they too are specific for each patient.¹¹

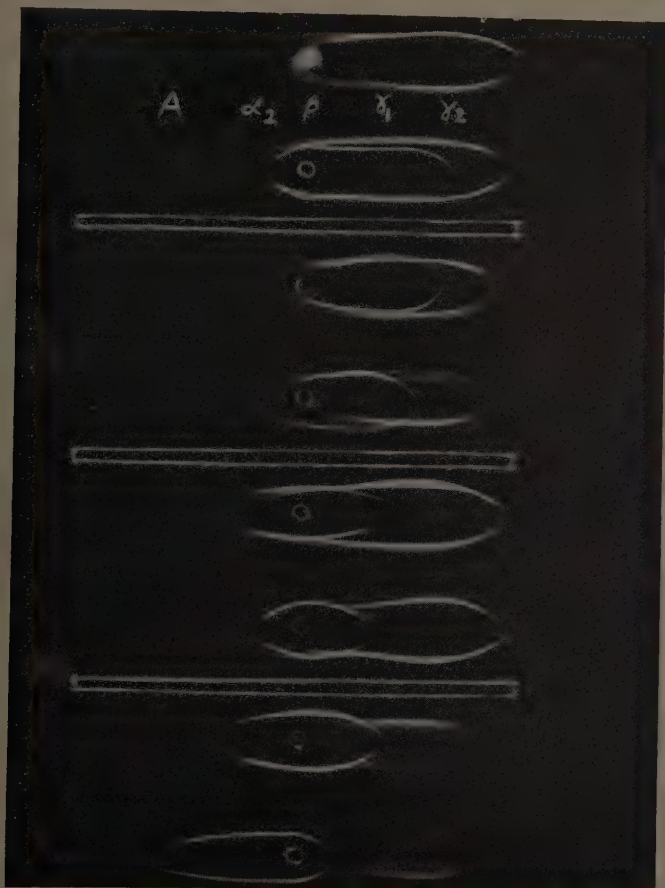


FIGURE 13. Immunoelectrophoresis of normal serum (*top*) and seven serums from patients with Waldenström's macroglobulinemia. Note the variability of mobility of MGW (the normal macroglobulin does not show up with these strong antisera).

There have been reports of sera containing macroglobulins with corrected sedimentation constants considerably lower than 17S to 19S.^{31,32} (The S values in the clinical literature usually are not corrected for concentration effects, that is, not extrapolated to zero concentration, and consequently they are frequently too low.) For these sera European literature has coined the term, "atypical macroglobulinemia."³² We have studied the abnormal globulin from one such serum* in detail. The electrophoretic pattern of this

* This serum was kindly supplied by William J. Hammack of the University of Alabama Medical Center, Birmingham, Ala.

serum is illustrated in FIGURE 15. The abnormal protein forms a sharp peak with a mobility of a γ_1 globulin, suggesting either multiple myeloma or macroglobulinemia. In the ultracentrifuge, 2 rapidly sedimenting components are apparent (FIGURE 16); their sedimentation constants (S_{20w}) are 9.6S and 11.9S (values not corrected for concentration). Therefore the diagnosis of Waldenström's macroglobulinemia is unlikely. The abnormal globulin did not react with antisera specific for γ_1 macroglobulin, but did react with antisera against γ_2 globulin. The purified globulin formed two lines of precipitate (FIGURE 17) and the curvature of these lines, away from the antibody cup, indicates that the antigens had diffusion coefficients less than that of 7S γ_2 -globulin.

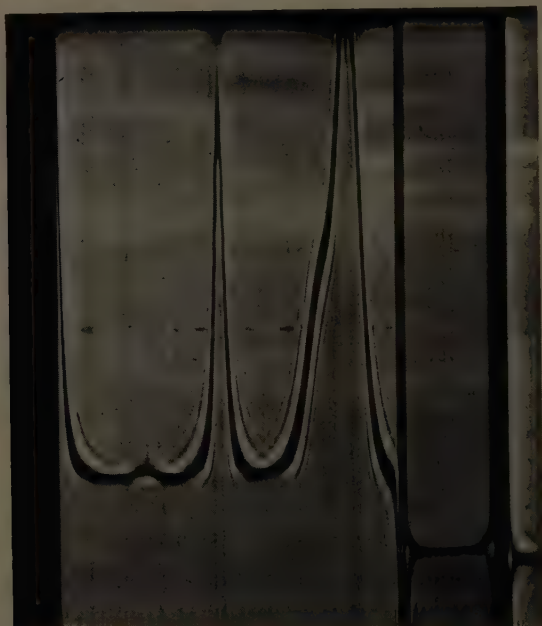


FIGURE 14. Ultracentrifuge pattern of serum from patient with MGW. Left to right: 26S, 19S, 7S, and 4S.

These two components together contained most antigenic determinants found in normal γ_2 globulin (FIGURE 18), but the antigen forming the lines closest to the antibody cup was antigenically deficient. From these immunological data we conclude that the atypical macroglobulins are polymers of the normally occurring 7S γ_2 -globulin. These polymers cannot be dissociated with mercaptoethanol (FIGURE 18, cup B), as can the MGW.³³ The abnormal protein(s) formed precipitates with aggregated human Fraction II (Cohn) and with antigen-antibody complexes prepared from rabbit antiovine serum albumin and bovine serum albumin in antigen excess (FIGURE 19). That the abnormal protein(s) are indeed the ones that were involved in this reaction is apparent because the zone formed on immunoelectrophoresis by the abnormal protein with anti- γ serums has the mobility range of the zone produced by aggregated Fraction II or the antigen-antibody complex and the patient's serum.

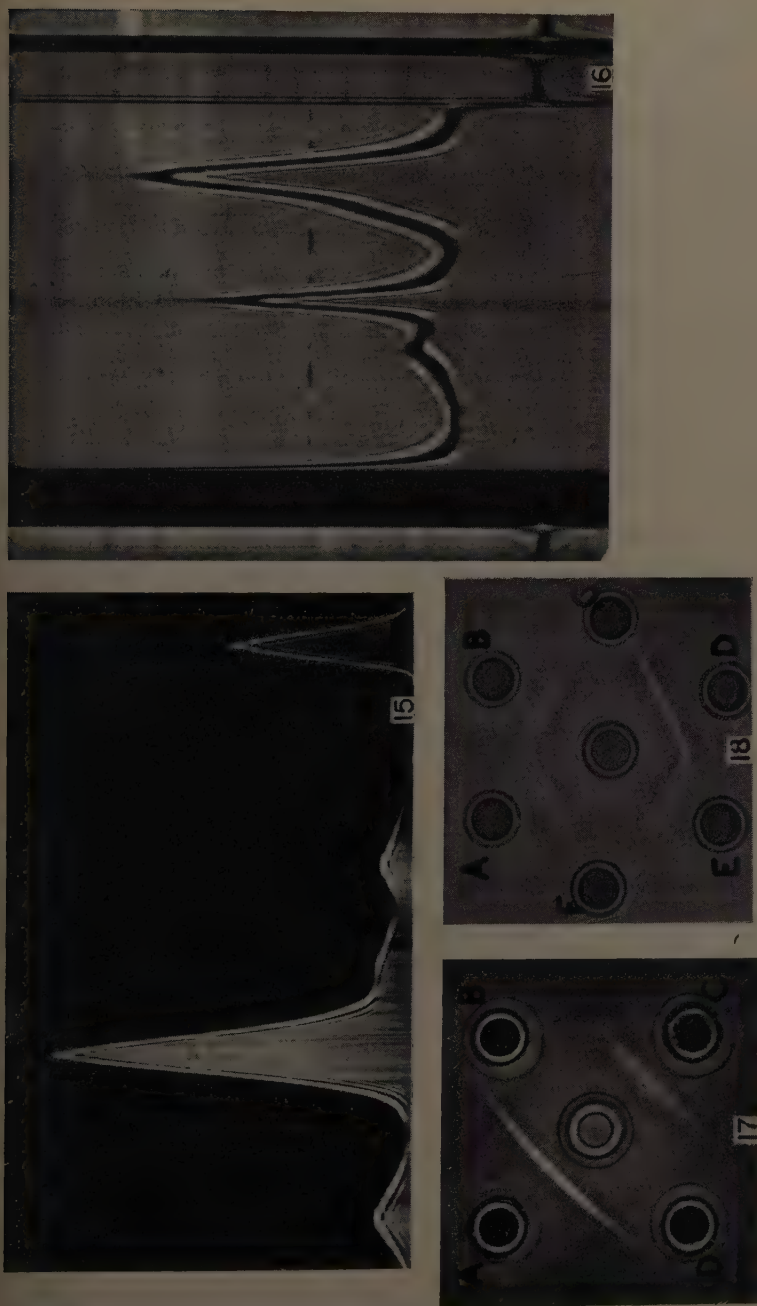


FIGURE 15. Moving boundary electrophoresis of serum from patient with atypical macroglobulinemia. Descending limb.
 FIGURE 16. Ultracentrifuge pattern of serum from patient with atypical macroglobulinemia. Left to right: 11.9S, 9.6S, 7S and 4S.
 FIGURE 17. (Center) anti- γ_2 -globulin sera; (A) γ_2 -globulin; (C) atypical macroglobulin, purified; (B and D) empty.
 FIGURE 18. The inertness of atypical macroglobulinemia to mercaptoethanol. (Center) anti- γ_2 -globulin sera; (A and E) purified native atypical macroglobulin; (B) purified atypical macroglobulin treated with mercaptoethanol and iodoacetate; (D) γ_2 -globulin; (C and F) empty.

The fact that these atypical macroglobulins produce precipitates with both the aggregated human Fraction II and the antigen-antibody complex is of some interest, since this kind of reactivity, for example in rheumatoid arthritis, is usually considered to be mediated by 19S (or 22S) globulins.^{30,34}



FIGURE 19. The immunoelectrophoretic properties of atypical macroglobulin and its reactivity with aggregated Fraction II and antigen-antibody complexes.

(Top) normal serum against anti- γ -globulin sera; (center) atypical macroglobulinemia serum against anti- γ -globulin sera; (bottom) atypical macroglobulinemia serum against antigen-antibody complex and against aggregated Fraction II.

DISCUSSION

The data presented in this paper emphasize the production of abnormal serum proteins in such diseases as multiple myeloma and macroglobulinemia of Waldenström. Some investigators maintain that these serum proteins are only quantitatively abnormal,^{18,23} according to them the same proteins may be present in normal serum, but at concentrations that may be too low to be detected with present methods. For the last few years our laboratory has

attempted to demonstrate myeloma proteins in normal serum. These efforts have led to the production of antisera capable of demonstrating the antigenic heterogeneity of normal γ_2 globulin. With them it has been possible to show that γ_2 globulin is a family of at least two closely related but not identical molecular species. (Some workers^{14,15} have suggested that the two lines formed with such antisera are due to one molecular species having several antigenic determinants, but our own data, to be published elsewhere, make this explanation unlikely.) Moreover, we have developed methods³⁵ for demonstrating forms of antigenic microheterogeneity that are beyond the resolving power of immunoelectrophoresis or the double gel diffusion technique of Ouchterlony. Nevertheless, we have as yet been unable to support the hypothesis that myeloma globulins are elevated normal serum proteins.

From immunological studies it seems that MM globulins lack some of the antigenic determinants of normal γ globulins; on the other hand, each myeloma globulin contains unique antigenic determinants. This apparent "loss" of normal determinants and the acquisition of abnormal determinants may be explained as follows. γ Globulin contains a limited number of antigenic determinants capable of eliciting an immune response in the rabbit. These determinants have a characteristic chemical configuration that provides them with their antigenic specificity, and they are duplicated by normal members of the plasma cell family. When the plasma cells become malignant, irregularities in γ -globulin synthesis may occur. Instead of synthesizing a γ globulin containing the characteristic chemical configurations, a protein is formed on which some of these configurations have been altered. This alteration may change the antigenic specificity of the protein so that the antibody against the original will no longer react with the altered determinant; that is, the myeloma protein has "lost" an antigenic determinant. Since the number of antigenic determinants of normal γ globulin is limited, it follows that the number of immunological groups in which myeloma globulins may be placed with antisera against γ globulin is also limited. However, the number of new chemical configurations that may be formed when protein synthesis goes astray is almost infinite, and consequently it is not surprising that each MM globulin (and MGW) is antigenically specific.

Our interest in multiple myeloma and macroglobulinemia stems from studies on intracellular antigens of normal and malignant cells. During this work it soon became apparent that little was known about intracellular proteins, and that methods for their isolation were still rudimentary. Consequently we turned to diseases in which the plasma cell became malignant, since it was known that in these diseases a cellular protein could be isolated in large quantities and purified to a high degree. Moreover, considerable biochemical and physicochemical information was already available about these proteins and their normal counterparts. The study of the various γ globulins served therefore as a model system for future antigenic studies of tumor cells. In collaboration with Daniel G. Miller we have applied the experience gained with the model systems to an immunologic analysis of leukocyte antigens. One of these antigens, extracted from the leukocytes of different patients with granulocytic leukemia, has an electrophoretic mobility that varied from γ globulin to α globulin, a finding that is reminiscent of the variability encountered with

MM globulins and MGW globulins. The variability is illustrated in FIGURE 20, in which leukocyte extracts of 7 patients with granulocytic leukemia were subjected to immunoelectrophoresis. The antiserum was specific for intracellular leukocyte antigens and reacted strongly with only one of them. The question whether these leukocyte antigens have lost antigenic determinants or acquired new specificities as yet cannot be answered.



FIGURE 20. Immunoelectrophoresis of extracts from leukocytes of patients with chronic granulocytic leukemia. Antiserum against granulocytes. *Top* and *bottom*: human serum against anti- γ -globulin serum.

SUMMARY

Plasma contains a family of functionally and structurally related proteins which Heremans has designated as immunoglobulins. In multiple myeloma, macroglobulinemia of Waldenström, and atypical macroglobulinemia, quantitative and qualitative abnormalities involving the three main components of the immunoglobulins may occur. These three main components are γ_2 globulin, γ_1A (β_2A) globulin, and γ_1 macroglobulin (β_2M). In multiple myeloma, the first two components, each of which comprises at least two antigenic species, may become altered; in macroglobulinemia of Waldenström, the γ_1 macroglobulin becomes abnormal and, in atypical macroglobulinemia, the γ_2

globulins seem to become polymerized. The abnormalities of these proteins manifest themselves in many ways: electrophoretic homogeneity, the presence of cryoglobulins, globulins with unusual electrophoretic mobilities, and/or sedimentation constants. Immunologically the pathological proteins lack some of the normal antigenic determinants and contain determinants that make them unique for each patient.

The large amounts of apparently abnormal globulins in the serums of patients with such malignancies as multiple myeloma or macroglobulinemia of Waldenström make these diseases good model systems for the study of abnormal protein synthesis in cancer. The extension of these models to the intracellular proteins of leukocytes has shown that they too may produce proteins that are abnormal in at least one respect, that is, electrophoretic mobility as measured by immunoelectrophoresis.

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Part III. Immunologic Phenomena and Alterations of Plasma Proteins in Infectious Disease

ANTIBODY PROTEIN SYNTHESIS *IN VITRO*

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The question of whether antibodies can be synthesized entirely outside the animal body has been controversial since Carrel and Ingebrigtsen in 1912¹ reported the formation of hemolysins to goat erythrocytes in 4 of 17 trials using guinea pig bone marrow and lymph nodes. No titers were reported, and this work could not be confirmed by Sato.² In 1913 Przygode³ incubated *Salmonella typhosa* with rabbit spleen and found agglutinins after 9 days in culture. Schilf⁴ mixed *Vibrio cholerae* with either rabbit or guinea pig spleens and demonstrated antibody by means of the Pfeiffer phenomenon in 10 of 13 trials. Höpken⁵ recently obtained 3 positive results in 49 trials in a true repeat of the work of Carrel and Ingebrigtsen.

On the negative side Parker⁶ was unable to demonstrate hemagglutinins to sheep erythrocytes in rabbit spleen cultures. Hwon⁷ could not detect antibody to diphtheria toxoid in rabbit spleen cultures. Sterzl and Rychlikova⁸ failed to demonstrate agglutinins to *Salmonella paratyphi B* in both rabbit spleen and bone marrow cultures.

Experimental

The methodology and experimental designs used in our experiments to date have been published by Stevens and myself.⁹⁻¹¹ In general, 2 systems have been used. The first of these utilized diced rabbit spleens maintained on the surface of a maintenance medium developed by Trowell¹² and modified by us.⁹ Little or no cellular replication occurred in this system and emphasis here has been on the early formation of antibody. The second system made use of replicating cells derived from peritoneal exudates of rabbits taken 3 days after the intraperitoneal injection of sterile heavy mineral oil. These cells, about 75 per cent monocytes, were cultured in roller tubes in 75 per cent medium 199 and 25 per cent normal rabbit serum. In both types of experimental systems, antibodies to the antigens used, bovine gamma globulin (BGG) and casein were assayed either by the Boyden hemagglutination technique¹³ or my modification of it.¹⁴

For the purposes of discussion the following definitions are used: a primary antibody response was that obtained after a single injection of the specific antigen, usually in a 40-mg. dose. A secondary antibody response was that obtained after the second injection of specific antigen 1 to 2 months after the first. The first dose was usually 40 mg.; the second was usually 4 mg. A hyperimmune antibody response was that obtained after multiple doses over a 3-week period.

Results

The results obtained in the early experiments that led to the study of the completely *in vitro* system are given in FIGURES 1 and 2. FIGURE 1 presents data for 5 groups of 5 rabbits given 1 intravenous injection of alum-precipitated BGG (AP-BGG). A series of nearly parallel curves of antibody titers was obtained in culture fluids of spleens removed 2, 4, 12, and 24 hours after primary injection.

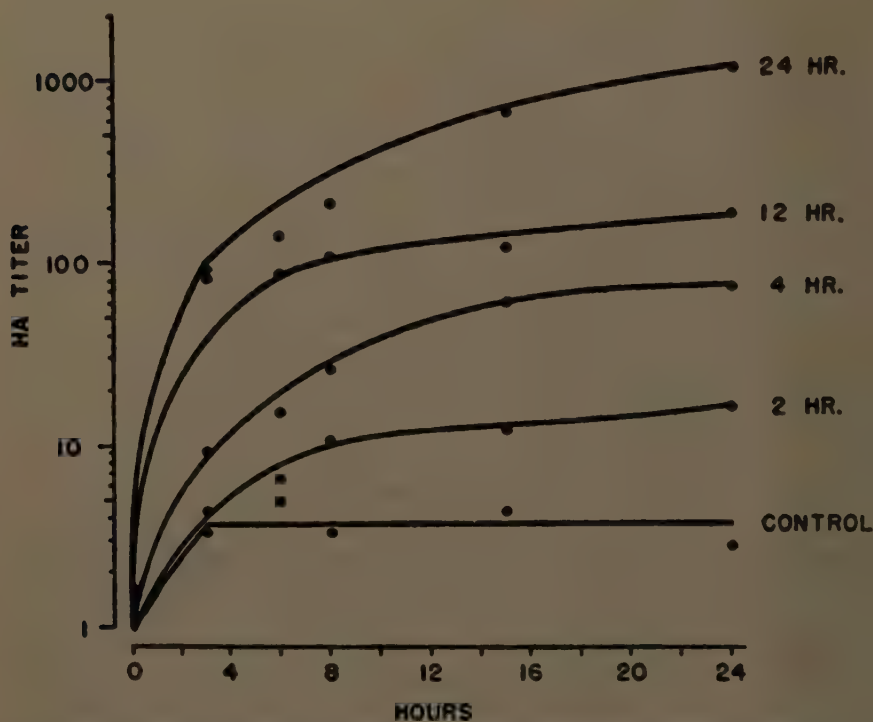


FIGURE 1. Primary response. Geometric mean hemagglutinin titers in culture fluids at various times of *in vitro* incubation. Column of figures at right shows the time of removal of spleens after injection of 40 mg. AP-BGG. Reproduced by permission of the *Journal of Immunology*.

The enhancing effect of the purified endotoxin of *S. typhosa* on the primary response to BGG was studied as follows: one group of 5 rabbits was given 40 mg. of AP-BGG intravenously. A second group of 5 animals was given 10 μ g. of endotoxin 24 hours before the same dose of AP-BGG. The third group of 5 was given 10 μ g. of endotoxin simultaneously with 40 mg. of AP-BGG. All rabbits were sacrificed 2 hours after injection of antigen and their spleens cultured in the usual manner. Geometric mean antibody titers for the 3 groups are given in FIGURE 2. It may be seen that when endotoxin was given 24 hours in advance of the antigen (BGG), approximately an eightfold increase in antibody titer was attained in the culture fluids 24 hours after *in vitro*

incubation. On the other hand, when the same amount of endotoxin was given together with the antigen in a single injection, no enhancement occurred.

For all the studies involving a completely *in vitro* synthesis of antibody, diced spleens from rabbits given 10 μ g. of endotoxin intravenously 24 hours

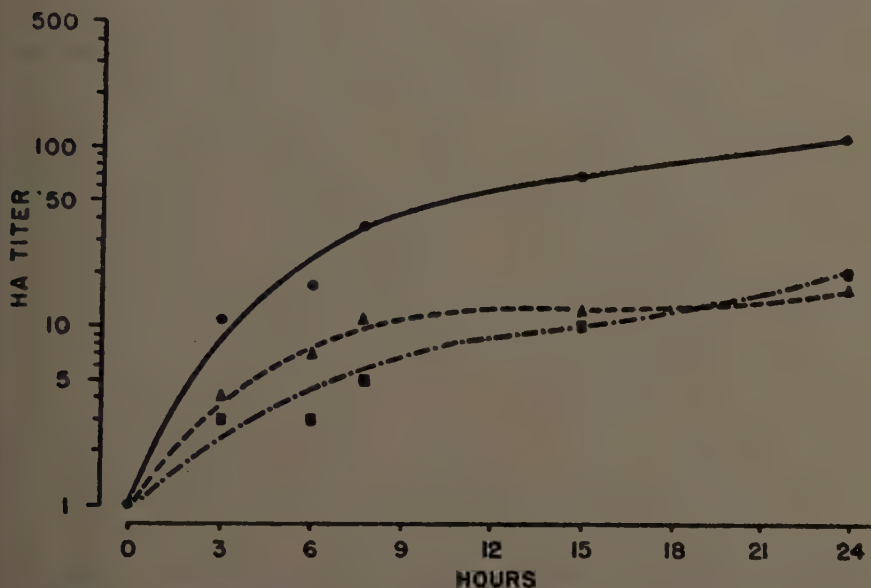


FIGURE 2. Effect of endotoxin (ET) on the primary response. Geometric mean hemagglutinin titers in culture fluids. All spleens removed 2 hours after 40 mg. AP-BGG. ●—● ET 24 hours before AP-BGG. ▲---▲ AP-BGG only. ■-·-·■ ET with AP-BGG. Reproduced by permission of the *Journal of Immunology*.

TABLE 1

GEOMETRIC MEAN HEMAGGLUTININ TITERS IN POOLED FLUIDS AND EXTRACTS AND THEIR SPECIFIC NEUTRALIZATION

	Control				BGG in Diluent				Casein in Diluent			
	Fluid		Extract		Fluid		Extract		Fluid		Extract	
	BGG	Cas.	BGG	Cas.	BGG	Cas.	BGG	Cas.	BGG	Cas.	BGG	Cas.
GMT	24	3	21	7	<2	3	5	7	12	<2	12	3
<i>t</i>	11.4		4.3		5.2		2.0		8.4		5.5	
<i>p</i>	<0.001		<0.001		<0.001		>0.05		<0.001		<0.001	

before sacrifice were incubated at 37° C. in modified Trowell's medium (TACPI) containing either 0.5 mg./ml. or 1 mg./ml. of BGG or casein. After washing, the tissues were planted as usual and culture fluids and tissue extracts assayed for antibody.

The results given in TABLE 1 represent mean titers of frozen pools from 1, 2, and 3 day cultures of the spleens of 9 rabbits. The hemagglutination inhibi-

tion tests were carried out by dissolving 0.025 mg./ml. of BGG or casein in 1 per cent normal rabbit serum in 0.85 per cent NaCl and using these as the diluents. While casein caused a reduction in the mean titer, this reduction was not significant and was slight compared with the effect of the specific antigen BGG. Student's "*t*" test was applied to the titration values, and the term "significant difference" denotes a "*p*" value of <0.001 . It may also be seen that casein did not evoke antibody formation under these conditions as all titers to casein were in the nonspecific range.

Early experiments showed that TACPI supplemented with normal rabbit serum had no enhancing effect on antibody synthesis over TACPI alone. It was felt that the serum from rabbits receiving endotoxin might enhance the response. Thus 5 pairs of rabbits were each given 10 mg. of endotoxin 24 hours prior to sacrifice. The spleens were kept entire at 1°C . for 1 hour until the sera had been prepared. Incubation with BGG and planting was carried out using the homologous and autologous serum with each of the 2 spleens.

TABLE 2
EFFECT OF 40 PER CENT AUTOLOGOUS AND HOMOLOGOUS SERUM

Treatment	Type response	GMT* 24-hr. fluid titer	
		Homologous	Autologous
10 μg . E.T.† 24 hr. before sacrifice	Primary <i>in vitro</i>	39	338
No E.T.	Primary <i>in vitro</i>	<2	<2
10 μg . E.T.	Secondary <i>in vitro</i>	24	256
No E.T.	Secondary <i>in vitro</i>	16	256

* GMT—Geometric Mean Titer.

† E.T.—Endotoxin.

The somewhat unexpected results are given in TABLE 2. The homologous serum from rabbits given endotoxin had no augmenting effect on the primary *in vitro* response, while autologous serum exerted a ninefold enhancement. The results obtained with secondary *in vitro* responses were quite different. In the latter situation, secondary spleens unexposed to endotoxin produced antibody *in vitro* and autologous serum increased the response. These results contrasted sharply with those from primary spleens in which no antibody was formed without endotoxin.

The system using monocytes was a replicating one. Early experiments were designed to ascertain the most suitable cell type for culture and the best medium. FIGURE 3 shows typical results obtained with peritoneal exudate cells, trypsinized bone marrow cells, and trypsinized splenic cells. It may be seen that cells from all 3 sources produced antibody for at least 6 days in 70 per cent medium 199, and 30 per cent normal rabbit serum.

The most consistent system for these studies was the peritoneal exudate cells (about 75 per cent monocytes) planted at 5×10^5 cells/ml. in roller tubes in 75 per cent medium 199, and 25 per cent normal rabbit serum.

A summary of the data obtained using this system is given in TABLE 3. In this system the cells from both primary and secondary rabbits produced

essentially the same amount of antibody over the same period of time. The cells exposed to BGG *in vitro* produced about the same amount as the primary and secondary cells but only for about two thirds of the time. The cells from

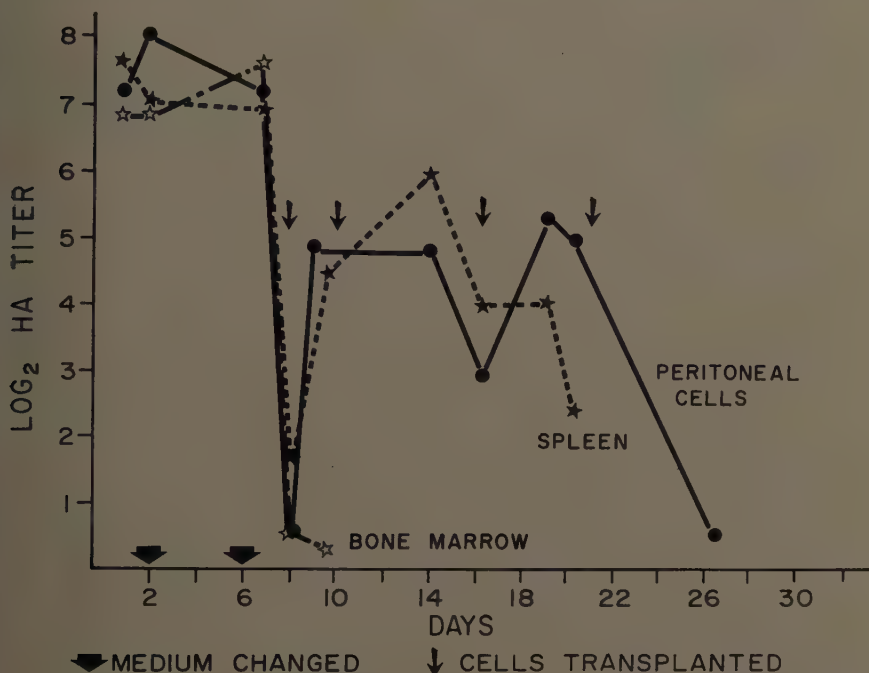


FIGURE 3. Formation of antibody *in vitro* by three tissues from a secondary rabbit. Reproduced by permission of the *Journal of Experimental Medicine*.

TABLE 3
COMPARISON OF GEOMETRIC MEAN HEMAGGLUTININATING TITERS OF CULTURE FLUIDS OF PERITONEAL EXUDATE CELLS

Antigenic experience	No. of experiments	No. of rabbits	GMT	Day antibody absent	No. of titrations
Primary	5	5	30	21	41
Secondary	5	5	32	23	28
<i>In vitro</i>	6	6	28	13	30
Hyperimmune	4	5	147	9	24
Primary and E.T.* <i>in vivo</i>	3	4	223	8	12
E.T. <i>in vivo</i> and antigen <i>in vitro</i>	3	5	97	6	12

* E.T.—Endotoxin.

hyperimmune animals produced about 5 times as much antibody as any of the other systems but for a period only about one half as long. In all of the experiments involving monocytes, the antibody titers remained quite constant in each system until the precipitous drops in titer at the end. When endotoxin was given to the rabbits together with antigen or when monocytes taken

from rabbits given endotoxin were exposed to BGG *in vitro*, the antibody response of the cells was of the same type as if the cells had been taken from hyperimmunized animals. That is, the titers were 5 to 7 higher than those of the homologous system without endotoxin but persisted for periods only one third to one half as long.

All attempts to demonstrate phagocytosis by either freshly harvested monocytes or cells in culture not in excess of one month were uniformly negative. This was true both for attempts with carbon particles as well as studies made with bacteria in the presence of opsonizing serum.

For studies of morphologic variation of the exudate cells, Kalter's quadruple stain¹⁵ was used. Monocytes in culture took on the angular, elongate shape of fibroblasts rather than the amorphous shape of macrophages. Thus Kalter's technique was thought to be most useful for studying monocytes to determine if they became fibroblasts as thought by Maximow^{16,17} and Bloom.¹⁸ All cells were stained with Kalter's quadruple stain and were photographed at $\times 450$.

The results of a typical series of cells stained *in situ* on 11×22 mm. cover slips are given in FIGURES 4 to 8. On first isolation, many of the peritoneal exudate cells were seen to have brick-red cytoplasm with eccentric horseshoe-shaped nuclei. These appeared to be typical monocytes; some contained oil droplets (FIGURE 4). After 3 days in culture, some cells were seen elongating, throwing out long spikes of cytoplasm. Nuclear changes involved rounding of the nucleus with the nucleoli becoming more prominent (FIGURE 5). After one week (FIGURE 6), little further change was noted, except that the cells were beginning to stain a dirty green. On the 24th day (FIGURE 7) the cells appeared mostly a dirty green. These cells contrasted quite sharply with kidney cells, derived from whole kidney, cultured for 24 days. The kidney cells stained a bright green (FIGURE 8), as expected for fibroblasts, and were smaller than the monocytes. In addition, the monocytes formed syncytia rather than sheets. Perhaps the dirty green stain taken on by the monocytes in late tissue culture reflects a tendency toward the common primitive mesenchymal ancestor of monocytes and fibroblasts.

Discussion

One of the most striking features of the experiments with monocytes was that there was no difference either in the magnitude or the duration of the antibody responses between cells taken from primary or secondary rabbits (TABLE 3). This shows both differences from and similarities to the spleen system. When the antigen was given *in vivo*, the secondary response in the spleen system was much greater than the primary response.⁹ However when the primary injection of antigen was given *in vivo* and the secondary contact was *in vitro*, the spleen showed no differences between primary and secondary *in vitro* contact.¹⁰

The magnitude of the response of normal monocytes exposed to BGG *in vitro* was essentially the same as that of cells from either primary or secondary rabbits. This differs from the results with spleen when both primary and secondary rabbits gave higher responses than did the completely *in vitro* system.^{9,10} In contrast, the primary and secondary monocyte series did

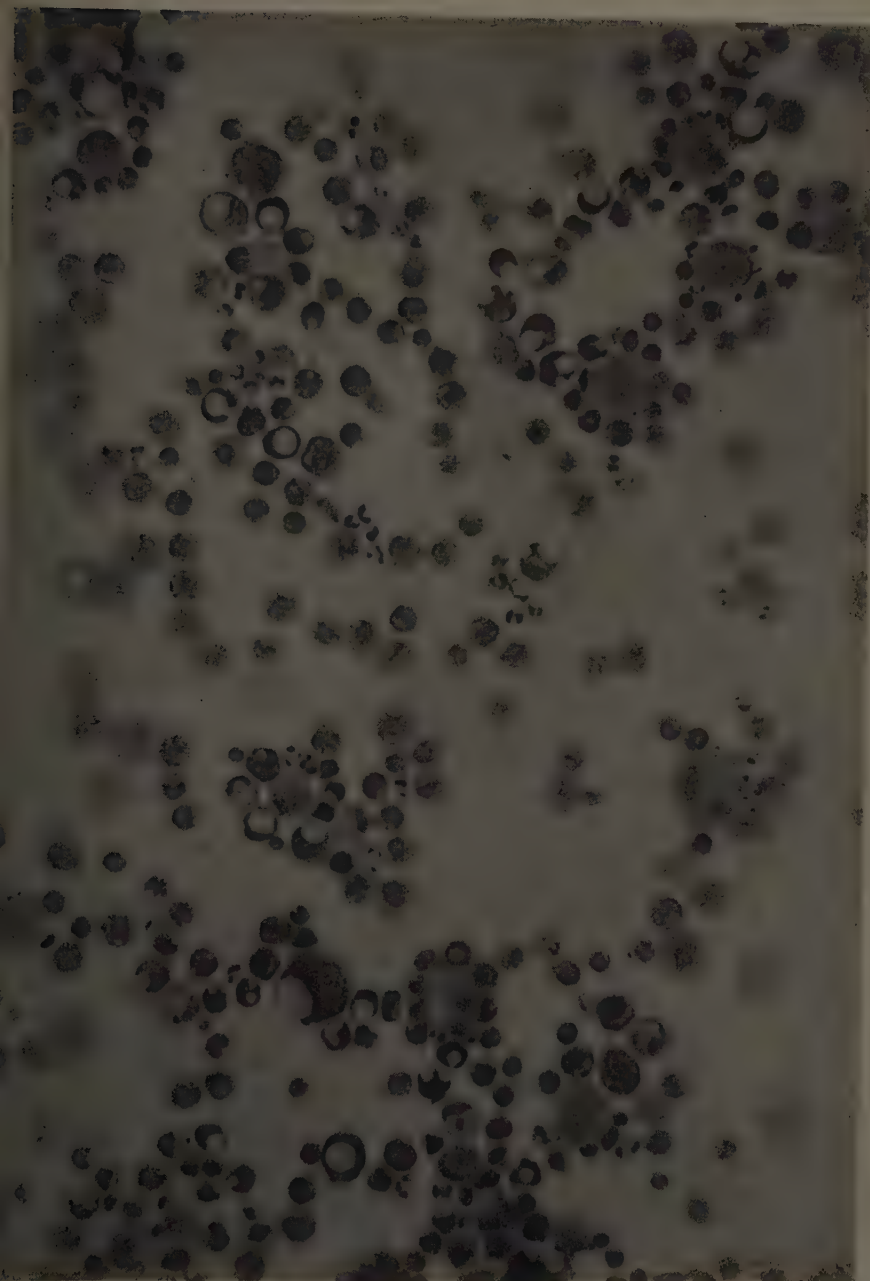


FIGURE 4. Freshly harvested peritoneal exudate cells taken 3 days after intraperitoneal injection of 100 ml. of sterile heavy mineral oil. Re-
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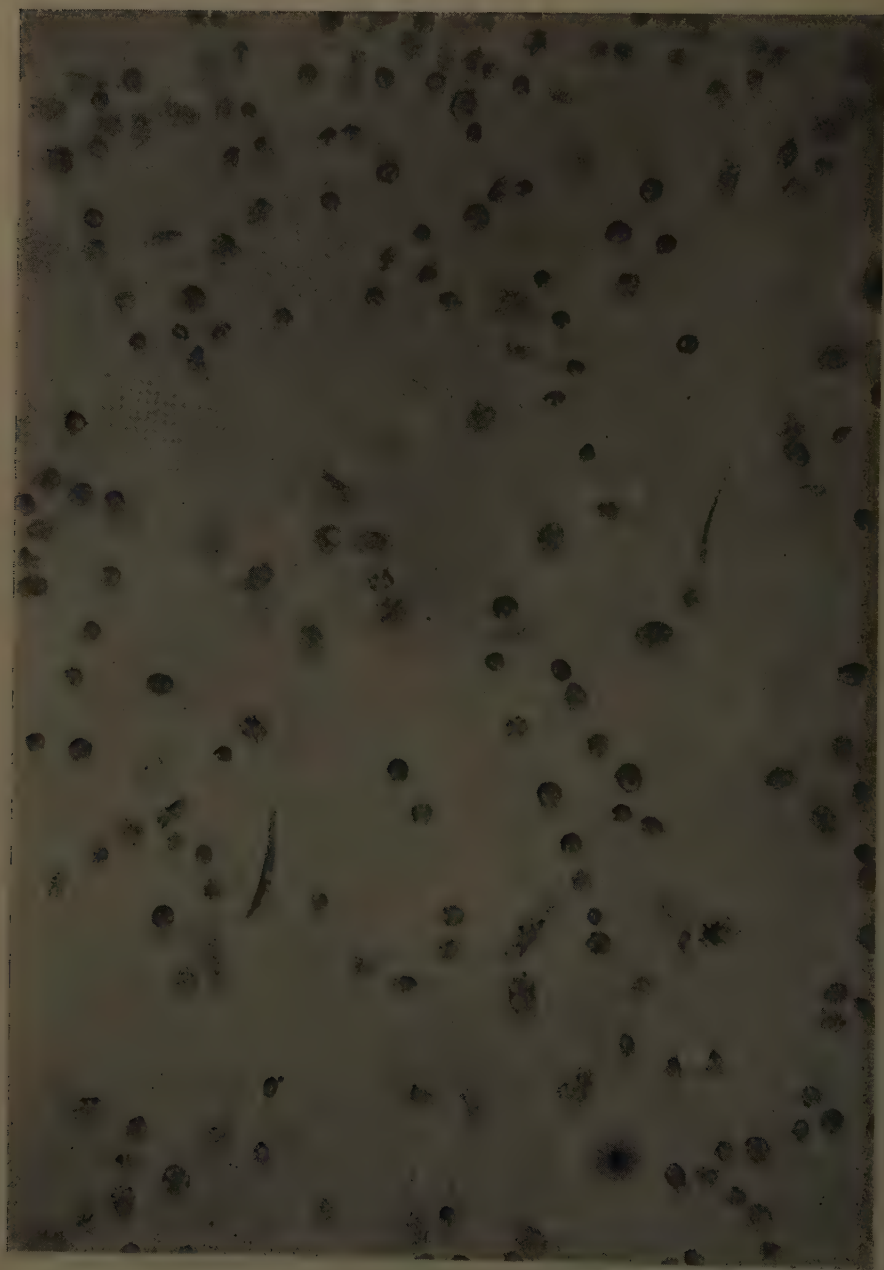


FIGURE 5. Peritoneal exudate cells after 3 days in culture. Reproduced by permission of the *Journal of Experimental Medicine*.

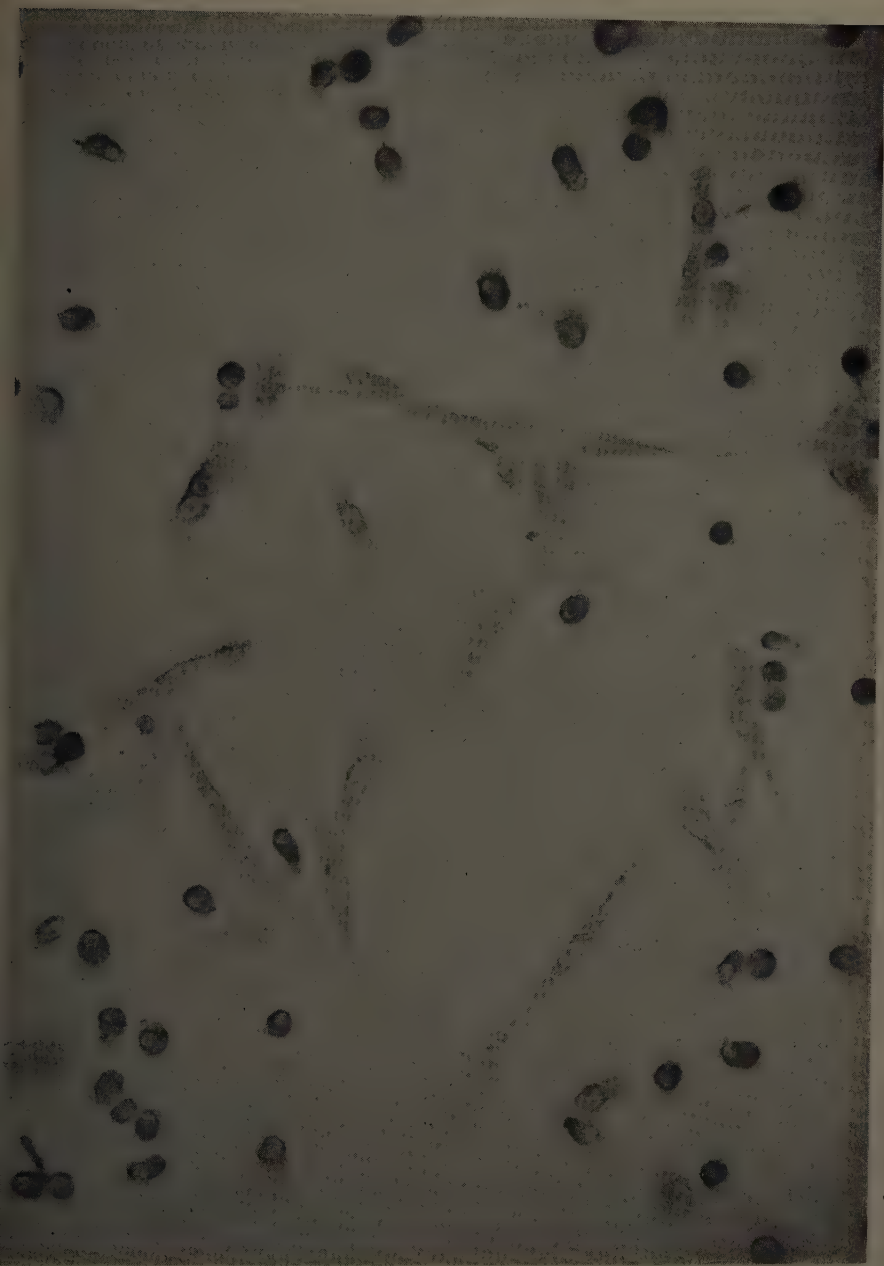


FIGURE 6. Peritoneal exudate cells after one week in culture. Reproduced by permission of the *Journal of Experimental Medicine*.

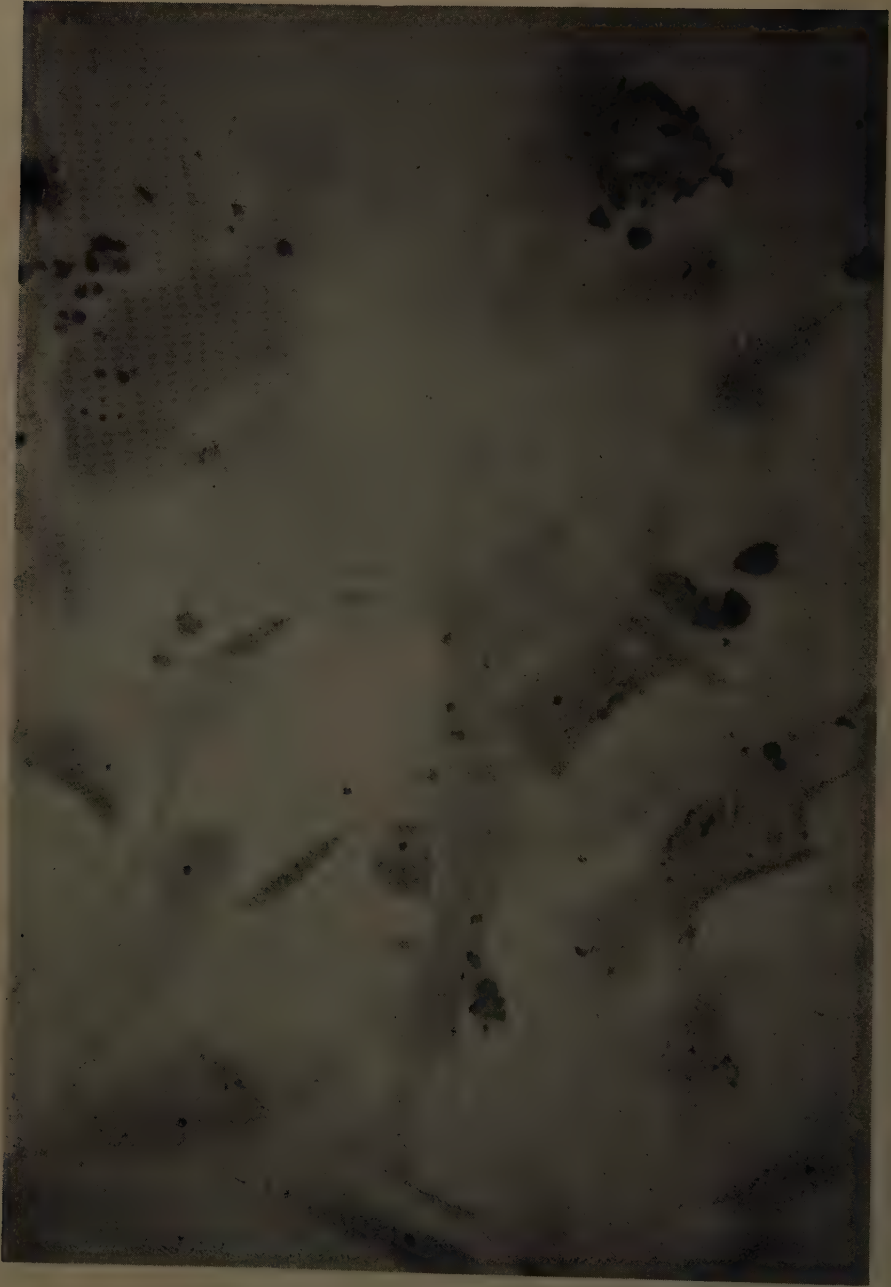


FIGURE 7. Primary peritoneal exudate cells after 24 days in culture. Reproduced by permission of the *Journal of Experimental Medicine*.

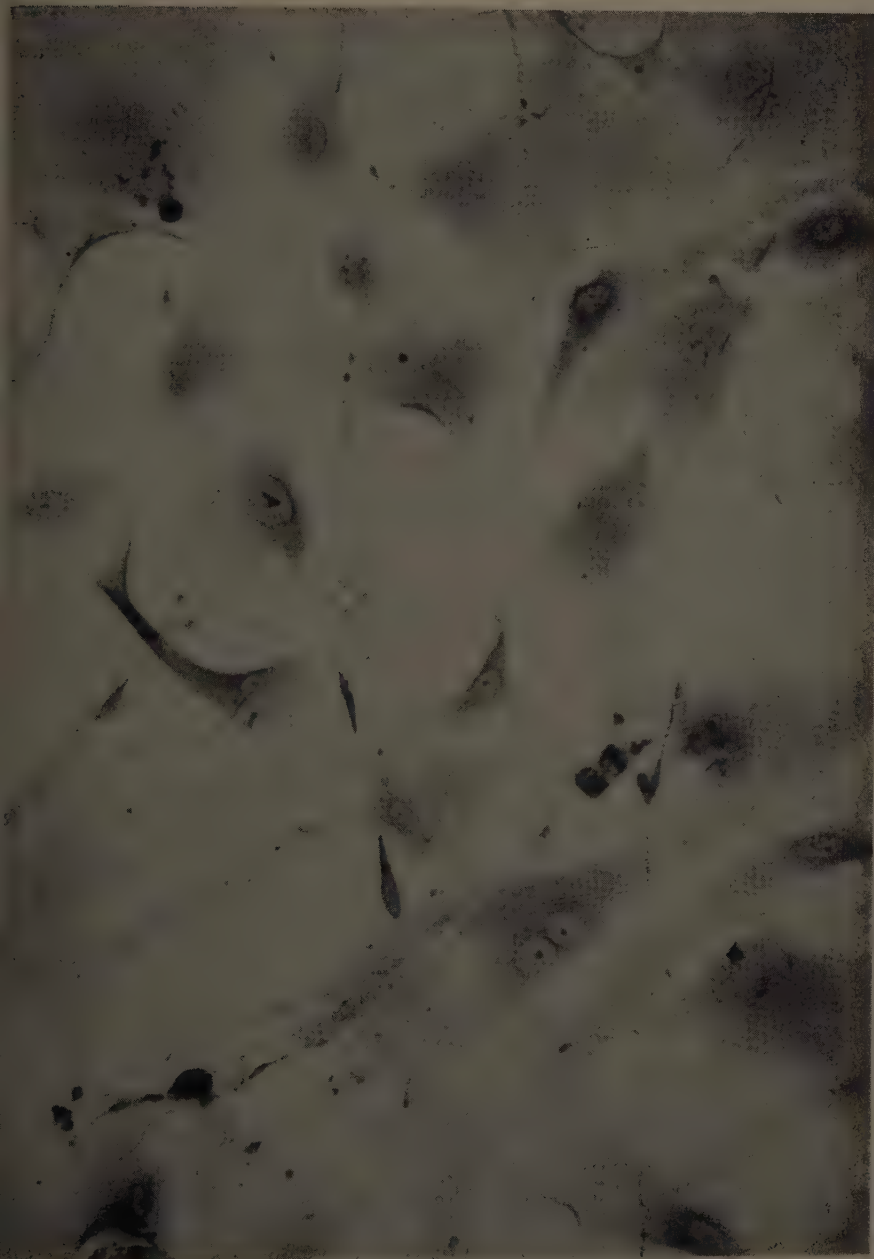


FIGURE 8. Primary trypsinized rabbit kidney cells after 24 days in culture. Reproduced by permission of the *Journal of Experimental Medicine*.

differ from the *in vitro* series in the duration of antibody formation in tissue culture; that is, three weeks versus two weeks respectively (TABLE 3).

It should also be kept in mind that pretreatment of the animals with endotoxin was not necessary to elicit antibody formation by monocytes after exposure to BGG *in vitro*. However, the mineral oil used to evoke the monocytes may have acted in a manner similar to endotoxin in that it made the cells responsive to *in vitro* stimulation by antigen.

Previously published data^{10,11} indicated that a critical dose of antigen was required for detectable antibody formation in both diced spleens and monocytes exposed to BGG *in vitro*. At all dosage levels, secondary spleens gave no better responses than primary spleens. These results, together with those of Sterzl,¹⁹ suggest either that the magnitude of the response is limited by an *in vitro* or neonatal environment,²⁰ or that the response, even of splenic cells, is monocytic in nature and perhaps limited.

A possible interpretation could be as follows: The monocyte system contains no lymphoid cells and shows no secondary response. The spleen system, on the other hand, contains both monocytic and lymphoid cells. While monocytes retain most of their functional abilities *in vitro*, lymphoid cells are much less hardy and rapidly lose these abilities *in vitro*. Hence when the spleen system is initiated *in vivo*, both monocytic and lymphoid responses are possible and secondary responses were observed when *in vivo* secondary spleens were placed in culture (FIGURE 1 and McKenna and Stevens).⁹ However when the antigen is introduced *in vitro* to either the virgin (that is, primary) or secondary spleen, the lymphoid cells are unable to react and only the monocytic response is obtained.¹⁰

The true primary response is considered to be a monocytic response, occurring over the first few hours to days. Lymphoid cells then became involved in an undetermined fashion and the majority of the antibody formed may be due to lymphoid cells. The division of the lymphoid cells then forms the basis of the secondary response. These mitoses could lead to a clonal-like picture, but they would not represent genetically determined clones as envisioned in the clonal theory.²¹⁻²³

In contrast to the lymphoid system, the monocyte-macrophage system of defense appears to have no secondary response; that is, no "memory" system. Monocytes are capable of reacting to antigen with a maximum effort once a threshold concentration of antigen obtains, irrespective of the previous antigenic experience of the animal from which the cells came.

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PLASMA PROTEINS IN BACTERIAL INFECTIONS

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For the sake of completeness, it seems necessary that someone in this monograph review the effects of bacterial infection on the plasma proteins. I have therefore undertaken this task. Although I have never worked directly in this field, I have tried to keep up with it, since many of the protein abnormalities seen in patients with cancer must be attributed to the presence of low-grade infections. I shall discuss changes in the plasma proteins: first, in acute bacterial infections, and second, in chronic infections, and then I shall review the literature on the C-reactive protein.

Acute Infection

In acute febrile infections such as pneumonia, tonsilitis, scarlet fever, rheumatic fever, and peritonitis, the principal changes in the plasma proteins are a decrease in albumin and an increase in the alpha globulins. These changes are not specific for infection; they also occur after injury or surgery.¹⁴ As suggested by Luetscher¹¹ a number of years ago, the picture is characteristic, not of any specific disease, but of the host's reaction to infection or injury; it appears to be related to the adrenal stimulation and the protein-catabolic reaction that follow trauma. This hypothesis is supported by the recent work of Grossman *et al.*,⁵ who studied the anabolism and catabolism of albumin following the injection of I¹³¹-labeled albumin. Some of their results are shown in TABLE 1. In their patients the initial rate of albumin degradation was 2.3 to 5.4 per cent per day. After the administration of hydrocortisone or corticotropin it rose promptly, and gradually reached a maximum of 4.6 to 8.6 per cent per day. The mean values increased from 4.3 to 6.2 per cent per day for the hydrocortisone group, and from 3.5 to 4.8 per cent per day for the corticotropin group. Since there was no change in albumin synthesis with hydrocortisone, the increase that followed the injection of corticotropin was ascribed to the secretion of anabolic adrenal hormones. One patient, with severe congestive failure, had a rate of albumin degradation of 3.8 per cent per day in the control period. During a severe respiratory infection the rate of degradation rose to 5.4 per cent per day, and albumin synthesis virtually ceased. Such an increase in the rate of degradation of albumin, accompanied by a decreased rate of synthesis, could account for the lowered levels of plasma albumin seen in infection. On the other hand, patients with adrenal insufficiency also show decreased albumin and increased alpha globulins, which are partially corrected by adrenal hormone therapy; clearly, much remains to be learned about the complex effects of the cortical hormones on protein metabolism.¹⁴

Both the decrease in albumin and the elevations in alpha-1 and alpha-2 globulins, first observed by moving-boundary electrophoresis,¹¹ have been confirmed by paper electrophoresis on larger numbers of patients. Lower alpha-

globulin values are obtained on paper, however, since this technique measures only the protein, and does not include the lipid and carbohydrate portions of these complex proteins. Also, the increases in disease are less marked. Some of the results of two large series are shown in TABLE 2. Jencks *et al.*⁸ compared 185 normal subjects and 187 patients with infectious disease. The only changes they regarded as significant (figures in italics) were a decreased albumin and elevated alpha-2 globulin; alpha-1 globulin was elevated, but only the

TABLE 1
ALBUMIN METABOLISM*

*No. patients	Period	Anabolism (%/day)	Catabolism (%/day)
5	Control	5.2	4.3
	Hydrocortisone	5.0	6.2
4	Control	4.4	3.5
	Corticotropin	6.1	4.8
1	Control	3.8	3.8
	Infection	<1	5.4

* Adapted from Grossman *et al.*⁵

TABLE 2
CHANGES IN THE SERUM PROTEINS IN BACTERIAL INFECTIONS, AS MEASURED
BY PAPER ELECTROPHORESIS*

	No.	Alb.	α_1	α_2	β	γ	T.P.
Normal mean $\pm 2\sigma$	185	69 60-77	2.9 1-5	7.3 4-10	9.0 5-13	12.0 7-17	7.3 5.9-8.7
Pulmonary tuberculosis	22	60	4.0	9.5	9.9	17.0	7.0
Pneumonia and bronchopneumonia	23	65	4.0	9.4	9.7	12.2	6.8
Pyelonephritis	11	60	4.7	10.0	10.5	15.3	7.0
Total infections	187	—	7+	+	0	15†+	

* Adapted from Jencks *et al.*⁸

† Number of patients with markedly elevated γ globulin.

values in pyelonephritis were regarded as abnormally high (TABLE 2). Graham *et al.*³ also studied 185 normal subjects. Their values for albumin and alpha-1 are similar to those of Jencks *et al.*, whereas their alpha-2 and beta values are lower, and their gamma globulin is higher (TABLE 3). The changes in 40 patients with bacterial infections (31 pneumonia, 4 meningitis, and 5 miscellaneous), were considerably greater than those seen by Jencks *et al.*,⁸ the average for albumin fell to 47 per cent. Since total protein was not reported, I have corrected the normal globulin values to the percentages that would have resulted if the only change had been the reduction in albumin. On this basis,

the patients' alpha-1, alpha-2, and gamma globulins are only slightly above the normal levels, and the beta globulin is reduced.

A better comparison may be made between the patients with bacterial infections and other patients in the same hospital, since both groups prior to admission had similar, poor nutritional backgrounds. Again I have calculated what the globulin values for the hospital patients would have been if they had lost enough albumin to reduce its level to 47 per cent. Compared with these, the patients with infections show substantial elevations in both alpha-1 and alpha-2 globulins.

Striking increases in alpha globulins are seen in the acute forms of the skin disease pemphigus; similar, but less marked, changes occur in the chronic forms of the disease.⁹

Acute infections are accompanied by little change in beta globulins. The values of Jencks *et al.*⁸ are well within their normal range, while those of Graham *et al.*³ are less than the corrected normal levels (TABLE 3).

TABLE 3*

	No.	Alb.	α_1	α	β	γ
Normal Mean $\pm 2 \sigma$	185	69 59-78	2.8 1-5	6.3 3-9	7.7 5-10	14.7 9-20
Normal $\times 1.7\uparrow$			(4.8)	(10.7)	(13.1)	(24.9)
Bacterial infections	40	47	5.1	11.1	10.4	25.9
Hospital patients $\times 1.25\uparrow$			(2.9)	(9.3)	(12.8)	(28.4)
Hospital patients	85	57	2.3	7.5	10.3	22.8

* Adapted from Graham *et al.*³

\uparrow Percentages of globulins if control albumin had dropped to 47 per cent.

In acute infections the plasma fibrinogen may be increased. The response may require several days to reach its maximum, which may be attained while fever and other symptoms are subsiding. When the infection is severe, a lack of fibrinogen response suggests liver damage.⁶

Acute infection usually causes little change in the gamma globulin of adults;⁴ Jencks *et al.*⁸ found markedly elevated gamma globulin in only 15 of 187 patients with various infectious diseases (TABLE 2). The elevated percentages of gamma globulins reported by Graham *et al.*³ can be attributed to the reduced albumin levels; there seems to be no great change in the concentration of gamma globulin (TABLE 3).

In children, however, the gamma globulin may increase either during, or shortly after, acute infection. In normal children under 11, Lubschez¹⁰ found elevated gamma globulin in 40 per cent of a group that had experienced various types of infection 1 month, or less than 1 month, earlier. In rheumatic children in apparent health the gamma globulin level was normal, except after respiratory illness; then it remained elevated for weeks to months, with or without the development of rheumatic fever. During acute rheumatic fever the gamma globulin was normal in the absence of antecedent illness.¹⁸

Chronic Infection

When an infection is prolonged, or produces a local lesion such as an abscess, from which antigenic material is absorbed, a marked rise in the gamma globulin may occur. Such diseases as tuberculosis, leprosy, and kala-azar, are most often associated with pronounced hypergammaglobulinemia.⁴

In one group of children with extreme hypergammaglobulinemia and recurrent infections, the primary cause appeared to be a congenital neutropenia; the elevated gamma globulin resulted from prolonged and repeated infections.⁴

The C-Reactive Protein

The changes in plasma proteins described above are quantitative: that is, they involve an increase or decrease in some component that was present before. The C-reactive protein, or CRP, on the other hand, has not been detected in normal human serum but appears only in response to disease or injury.¹⁴ Its name is derived from its ability to precipitate the somatic C-polysaccharide of pneumococcus.¹⁷ CRP is not antibody, however; it differs from antibody in several ways, as discussed below.

The CRP was first observed in the serum of patients acutely ill with pneumococcal pneumonia, but is also found in other acute bacterial diseases.¹ In acute rheumatic fever its presence is a sensitive indicator of rheumatic activity.¹⁴ When it occurs in serum, it may also be found in the serous fluids.¹⁹ It has been noted in the serum of monkeys with pneumonia.

In serum and in most serous fluids the CRP occurs in combination with lipid.¹⁹ It was first crystallized from a chest fluid, in which it occurred free of lipid;¹² before CRP from other sources could be crystallized, the lipids had to be extracted with chloroform. The final procedure involved fractionation with ammonium sulfate, precipitation by dialysis against 0.01 per cent CaCl_2 , extraction with chloroform, precipitation with C-polysaccharide, and crystallization from 0.75 saturated ammonium sulfate at 37° C.¹⁹ CRP forms symmetrical rhomboid crystals. It contains 14.66 per cent nitrogen and no phosphorus. It is soluble in distilled water. On moving-boundary electrophoresis at pH 7.45, the CRP has the mobility of a beta globulin. Its isoelectric point is at pH 4.82. In zone electrophoresis on starch it is found in the gamma region; it is interesting to speculate that this retardation might result from the formation of complexes with the starch. Its sedimentation coefficient at infinite dilution is 7.5 S.¹⁴

The CRP differs from antibody in several ways. The serum titer is maximal in the active stage of a disease, and decreases rapidly after the onset of convalescence; the reaction with C-polysaccharide requires calcium;¹ and CRP and antibody have different physical properties.^{12,19} Finally, the presence of CRP is not limited to pneumococcal infections but is noted in many other conditions. A positive test is found in many acute bacterial, protozoal, and viral infections, and in active rheumatoid arthritis. It is frequently positive in patients with metastatic cancer or myocardial necrosis, and in pregnant women, especially during labor.¹⁴

CRP can be detected by its ability to precipitate the C-polysaccharide in the presence of calcium, or by its ability to cause capsular swelling of pneumococci.⁷ The most sensitive and precise tests, however, are immunological.

Crystalline CRP is highly antigenic in the rabbit, giving rise to specific antibodies which show no cross-reaction with normal serum.¹² Both complement fixation and precipitin tests have been used;¹⁴ the latter may be rendered more accurate by the use of gel-diffusion methods.²

Although the source of CRP is unknown, some indirect evidence as to its origin has been obtained. Following surgery the CRP increases rapidly within the first two or three days, and then declines; the maximal level attained seems to be related to the degree of trauma. The fact that CRP does not appear until six or eight hours after the initiation of surgery suggests that it is not released from some bound form in the tissues but is rather a manifestation of activity of cells produced in early response to tissue injury, such as macrophages and polymorphonuclear leukocytes.¹⁵

Further evidence for the involvement of the reticuloendothelial system has come from studies on a similar substance, the Cx-protein, produced by rabbits. Intravenous injections of colloidal thorium dioxide (Thorotrast) will result in the appearance of Cx-protein, but in amounts that diminish with each successive injection. When the reticuloendothelial system is almost completely blocked very little Cx-protein can be elicited.¹³ After rabbits have been injected with immunological adjuvant their serum contains a new substance (Co-Cx-protein). Injection of Co-Cx-protein into normal rabbits elicits the appearance of Cx-protein. The Co-Cx-protein is found in the seromucoid fraction of serum. Injection of Co-Cx-protein 2 to 3 hours after intravenous injection of a nonlethal dose of Thorotrast is fatal, although normal seromucoid does not enhance Thorotrast toxicity.¹⁶

Summary

The changes in the plasma proteins in bacterial infections are of three types: a reaction to acute infection, probably mediated through the adrenal cortex; a reaction to chronic infection, correlated with antibody production; and the appearance of the C-reactive protein whose origin is unknown.

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SERUM PROTEIN AND ANTIBODY STUDIES IN VIRAL DISEASES: A REVIEW*

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INTRODUCTORY REMARKS

Fractionation by both physical separation and chemical precipitation methods of antibacterial, antitoxic, and antiprotein sera into their respective serum protein components has shown, in general, a decrease in the albumin/globulin ratio. This has been the result of a decrease in the relative and absolute amounts of the albumin component and an increase in globulins, particularly gamma globulins. The increase in the globulin fractions usually parallels the antibody titers as demonstrated by various serologic and immunologic tests.

Reports on the analyses of the protein composition of antiviral sera by similar physical and chemical fractionation procedures, in most instances, have not shown significant alterations in either the relative and absolute quantities of the individual serum proteins. This lack of significant change was adequately demonstrated in these instances in sera that possessed relatively high antibody titers as shown, usually, by viral-neutralization and complement-fixation tests. It has generally been understood that most of the antibody activity in these sera was present in the gamma-globulin fractions. There have also been reports in the literature on a number of antiviral sera that have shown alterations similar to those found in antibacterial, antitoxic, and antiprotein sera. Antibody studies were not conducted in most of these cases since immunologic and serologic testing methods had not been developed for the particular infectious conditions.

In any case the alterations observed have not been considered specific for any one infectious disease since the changes only measure the current status of the subject under study. One exception to this general rule has been reported in which natural infections of *Eperythrozoon* in the bovine species produce concurrent hypoglobulinemia, and the change is most evident in the gamma-globulin fraction.¹

The main purpose of this review is to consolidate experimental results on the subject and to present data and interpretations on the serum protein composition of antiviral sera and the various antibody studies that have been reported. Particular emphasis will be placed on the antibody studies conducted on separated serum fractions. Additional data from a limited number of reports dealing with the various classes of antibodies that are found in the protein fractions of antiviral sera will also be presented. Individual viral diseases based on their tropisms will be considered.

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PRIMARY STUDIES

An intensification of effort was made during World War II to fractionate plasma from normal human blood collected by the Red Cross.² Originally the objective was intended primarily for the separation of the albumin fraction that was to be used as a plasma substitute and as an aid in increasing colloid osmotic pressure in the blood. The by-products, the globulins, subsequently became available for further study and a broad program of antibody determinations was undertaken on these fractions.³

The fractionation scheme yielded gross fractions consisting of alpha, beta, and gamma globulins, and these fractions were designated as Fraction II + III. The material contained a large amount of antibodies reacting with a variety of pathogenic bacteria and their products and several viruses. These "normal" gamma globulins were further separated and concentrated in Fraction II by at least 15 to 30 times as compared to pooled whole plasma. Immunologic assays of Fraction II showed that it contained antibodies against many bacteria, especially for influenza A and mumps viruses. Although immunologic and serologic tests were made for the determination of antibodies to a limited number of other viral agents there was not a sufficient concentration of antibodies present to manifest a reaction. Fraction II, which was largely gamma-globulin, was found to contain complement-fixing antibodies for mumps and influenza A viruses, whereas the viral-neutralizing and hemagglutination-inhibiting antibodies for influenza A virus were also found in this fraction.

RESPIRATORY VIRUSES

Influenza

A study of anti-influenzal horse serum produced by massive inoculations of allantoic fluid from embryonating chicken eggs infected with the PR-8 strain of influenza virus was reported.⁴ Using initial electrophoretic separation of the serum protein components, it was found that the patterns were not greatly altered when compared to normal serum samples. Globulin fractions precipitated by various concentrations of ammonium sulfate were subsequently separated and the homogeneity checked by electrophoretic methods. It was found that the hemagglutination-inhibiting antibodies were associated with the slower globulin components, especially the gamma globulins. There was also a suggestion that the hemagglutination-inhibition titer approximated the planimetric units and area under the peaks of the separated gamma-globulin fractions after corrections for dilutions were made.

Infectious Bronchitis

Analyses of sera from chickens experimentally inoculated with infectious bronchitis virus yielded data indicating that a challenge dose of active virus 12 weeks after primary inoculation did not produce a significant change in the absolute quantities of total globulin or gamma-globulin. Although a substantial rise in neutralization index was observed above that due to the primary inoculation the absolute values for total globulin and gamma globulin were

minimum. Initial inoculation with virus and subsequent weekly bleedings of 20-ml. volumes from each bird produced a decrease in the albumin/globulin ratio, but the neutralizing antibody response to the primary dose was not impaired by the imbalance of the serum protein components. The large volume of blood withdrawn at weekly intervals was responsible for the increase in the amounts of globulins subsequent to primary inoculation.⁵

Newcastle Disease

Very little difference in the serum proteins was observed between normal chickens and chickens infected with Newcastle disease virus, although in some cases a slight increase occurred in the amount of gamma globulin. Electrophoretic fractionation of immune chicken serum yielded pure gamma globulin that was rich in viral-neutralizing and hemagglutination-inhibiting antibodies.⁶

Differences were observed in the amounts of globulins that were discernible in anti-Newcastle disease serum from chickens when two methods of analysis were employed. By paper-electrophoretic techniques no changes in the serum protein components were noted, although sodium-sulfate precipitation techniques showed that a rise of 20 per cent in the globulin fraction occurred. A suggestion was made that a relationship existed between the increased total precipitable globulins and the hemagglutination-inhibition antibody titer.⁷

Large quantities of immune sera were prepared in cattle by inoculation of formalinized and live Newcastle disease virus. The antisera were fractionated by low temperature alcohol precipitation methods and the fractions containing both the neutralizing and hemagglutination-inhibiting activity were separated. Neutralizing activity was found to be present in both the gamma-1 and gamma-2 globulins but only the gamma-1 fraction contained hemagglutination-inhibiting activity. Further fractionation of gamma-1 into gamma-1b showed that this new fraction possessed neutralizing activity but no hemagglutination-inhibiting activity. The gamma-1a fraction lacked both inhibiting and neutralizing activity as demonstrated by the viral-neutralization test in embryonating chicken eggs and the hemagglutination-inhibition test. It was inferred that the inhibiting and neutralizing activities may be functions of different antibodies since it has been generally accepted that the two antibody titers in the infected or immunized chicken are not reached simultaneously and are not maintained equally. It was also found that neutralizing antibodies persisted longer than hemagglutination-inhibiting antibody levels.⁸

NEUROTROPIC VIRUSES

Poliomyelitis

Considerable work on the protein composition of serum and plasma from poliomyelitis patients has been reported. Analyses by the moving-boundary method of electrophoresis of serum proteins from human patients in various stages of the disease did not show consistent or significant differences from the normal.^{9,10} A fast migrating component that was ahead of the albumin was found, and it was stated that this component was characteristic of the acute phase of the disease. Antibody studies were not conducted.⁹

Other reports have indicated contrary results and it has been demonstrated that the serum and plasma protein components are greatly altered. Using electrophoretic methods, it was found that the albumin/globulin ratio decreased roughly in proportion to the severity of the disease. The alpha-1 and alpha-2 globulins and fibrinogen showed an increase whereas the gamma globulins were normal, but the gamma-1 fraction decreased in amount as did the total serum protein content.¹¹ In the early stages of the disease the alpha- and beta globulins showed an increase while the albumin decreased.¹²

Chemical precipitation methods have demonstrated that no changes occur in the total serum proteins or albumin during the first three weeks of illness. In the chronic stages of poliomyelitis infection a slight but significant difference in the total serum proteins was found. Presumably this increase in serum protein content was associated with the prolonged immobilization periods involved in some severe chronic cases.¹³

Poliomyelitis infection in humans has attracted a significant amount of study on the beta disturbance as observed when serum is subjected to moving-boundary electrophoresis. The beta disturbance or spike was said to be specific in poliomyelitis sera although accurate interpretations were difficult.¹⁰ A further study of this phenomenon in poliomyelitis indicated that the beta spike was shorter than normal, as observed in the descending boundary,¹⁴ and that it sometimes decreased entirely.¹⁵ There was a greater incidence of occurrence of major changes in the beta spike in electrophoretic patterns of serum from poliomyelitis patients than in serum from normal individuals even when using different buffers. The beta spike has been likened to a turbid lipoprotein complex that is formed during electrophoresis. No correlation was found to exist between the changes observed in the disturbance and the severity or duration of the disease.¹⁵

An investigation of normal pooled adult human gamma-globulin preparations was conducted to determine poliomyelitis viral antibody distribution. The results showed that regardless of geographical origin or manner of preparation, neutralizing antibodies against 3 specific poliomyelitis virus types, namely (type 1) the Brunhilde strain, (type 2) the Y-SK strain, and (type 3) the Leon strain, were present. A further study of the distribution of neutralizing antibodies in the serum fractions demonstrated that these antibodies were present in the gamma-globulin fraction, although both the beta and gamma globulins prepared from the same pool of plasma possessed neutralizing antibody activity to about the same degree.¹⁶

Several attempts have been made to produce large quantities of anti-poliomyelitis viral horse serum for subsequent passive treatment.^{17,18} Although horse serum contains a relatively small quantity of euglobulin it was reported that serum from horses immunized with virus contained a large amount of euglobulin. Neutralizing antibody tests conducted in monkeys did not show any antibody activity in this fraction. It was later found that the neutralizing antibodies were concentrated in that portion of the globulin fraction that was on the borderline between the pseudoglobulin and euglobulin.¹⁸

Studies on serum proteins in cerebrospinal fluid by electrophoretic methods have shown that a component with the same mobility as gamma globulin is present after pleocytosis occurs in the fluid.¹⁹

Rabies

Separation of the globulins in antirabies horse serum by isoelectric precipitation yielded a gamma-2 globulin fraction in which most of the neutralizing activity resided. The purified antibody preparation was used further as a conjugate with a fluorochrome dye and it proved to be of diagnostic value for detecting rabies virus.²⁰

Neutralizing antibodies present in antirabies rabbit serum were found to be almost exclusively in the gamma globulin. Some nonspecific masking was observed with normal sera and other serum fractions separated in the Tiselius cell.²¹

Japanese B Encephalitis

Immunization of rabbits with Japanese B encephalitis virus and subsequent electrophoretic analyses of immune sera showed that although neutralization titers were high, as tested in mice, there were no changes in the serum protein patterns as compared with the normal.²² Most of the neutralizing activity was found to be present in the gamma-globulin fraction of material separated in the Tiselius cell.²¹

Western Equine Encephalomyelitis

Fractionation by ammonium sulfate of anti-Western equine encephalomyelitis rabbit serum showed that neutralizing antibodies were present only in the globulin fraction. Further analyses of these sera and separation of fractions obtained in the Tiselius cell showed that neutralizing activity was not only present in the beta and gamma globulins but also in the albumin. The whole serum electrophoretic patterns were not changed significantly.²³ Other reports stated that neutralizing antibody activity of immune rabbit serum resided almost exclusively in the gamma-globulin fraction separated in the Tiselius cell,²¹ although some activity was also present in the beta globulin.²² Albumin did not possess antibody activity²² but some nonspecific inhibition toward the virus by normal whole sera and other separated serum fractions was demonstrated.²¹

Eastern Equine, Venezuelan Equine, and St. Louis Encephalitis

Immune sera were prepared in rabbits using these three viruses. The sera were separated by the moving-boundary method of electrophoresis, and the serum fractions were removed from the Tiselius cell and analyzed for neutralizing antibody. Antiviral activity was found to be almost exclusively in the gamma fraction for Eastern equine and St. Louis encephalitis viruses²¹ whereas antibody activity for Venezuelan equine encephalitis resided in both the beta and gamma globulins.²²

African Horsesickness

Using an apparatus similar to the Tiselius cell Polson²⁴ was able to remove fractions of immune serum prepared in the horse at various levels after separation by electrophoresis. Although no specific identification was given to the separated materials it was found that the fraction containing neutralizing antibodies had an isoelectric point similar to that of beta globulin.

NEOPLASTIC

Avian Leukosis

Significant differences in the serum or plasma protein composition of chickens infected with the leukosis complex have not been demonstrated by paper-electrophoretic or ultracentrifugal methods.²⁵ The use of the moving-boundary method of electrophoresis in the analyses of serum obtained from infected chickens demonstrated a new component, namely the L component. This fraction, which represented approximately 10 per cent of the total serum protein, was found in the sera of chickens with various manifestations of leukosis. The L component possessed a mobility of 2.55×10^{-5} cm.² sec.⁻¹ volt⁻¹. A decrease in the amount of albumin and an increase in the alpha globulin was noted.²⁶

In still another study, the L component was not observed in chicken sera even in birds that showed gross manifestations of the leukosis complex at necropsy. It is possible that this component may be an artifact similar to that observed in the patterns from the ascending limb of the electrophoresis cell and that it may be comparable with the gamma anomaly of chicken sera in the pattern of the descending limb. The L component, or a component similar to it was observed in the ascending pattern of sera from chickens that did not show evidence of leukosis at necropsy.⁵

PANTROPIC AND VISCEROTROPIC VIRUSES

Lymphogranuloma Venereum

Antibody studies have not been conducted during serum protein analyses of sera from lymphogranuloma patients. Inferences have been made that the hyperglobulinemia observed as determined by the Tiselius moving-boundary method²⁷⁻²⁹ may have been due to antibodies.²⁷ In general, all globulins increased and albumin decreased in amounts during the acute stages of the disease. Treatment with sulfa drugs apparently destroyed the virus and, since the infectious stimulus was absent, the gamma-globulin level returned to normal.^{29,30} Chemical fractionations have also resulted in the observations that a hyperproteinemia exists and that there is also an increase in the globulin concentration.³¹⁻³⁴

Rinderpest

Limited antibody studies in bovine rinderpest viral infections have shown that the increase in the globulin content was due mostly to an increase in the euglobulin although in a few instances an increase was also observed in the pseudoglobulin, concurrently.³⁵ Hyperimmunization of cattle by the intramuscular route produced an increase in the total serum protein content, total globulin, and euglobulin.³⁶ When cattle had virus administered by the intraruminal route the changes were similar to those changes observed in the sera of cattle given virus by the intramuscular or subcutaneous routes although most animals showed insignificant changes. Although the globulins increased, and this was probably indicative of antibody formation, the results supported the

view that antisera made by the intraruminal method were inferior to antisera prepared by other methods.³⁶

In the goat, administration of rinderpest virus produced an increase in the globulins similar to those observed in cattle, but there was more pseudoglobulin and less euglobulin present. It was suggested that in the goat the antibodies against rinderpest virus were distributed over a wider serum protein range than in the bovine species.³⁷

Equine Infectious Anemia

Using both chemical fractionation and moving-boundary methods of separation it was found that horses infected with equine infectious anemia showed, generally, a decrease in the albumin/globulin ratio^{38,39} and a rise in the total serum protein content shortly after infection.³⁹ The changes in the fractions appeared to be related to the severity of the disease or to the number of acute phases.³⁹ One report appeared which stated that no great change occurred in the serum proteins of the horse.⁴⁰

Infectious Mononucleosis

There is general agreement in the results of two investigations on the composition of sera from human patients showing clinical evidence of infectious mononucleosis.^{41,42} There were some increases in the amounts of alpha and beta globulins and a marked increase in the gamma-globulin concentrations whereas the concentration of albumin decreased. Although heterophile antibodies were found predominantly in the gamma-globulin fraction the increase in the concentration of this globulin was not due to antibody formation but most likely to hepatic dysfunction and subsequent decrease in the concentration of albumin. That this globulin change was not due to antibody formation was demonstrated by the fact that no changes occurred in the globulins of patients' sera after absorption with sheep erythrocytes.⁴²

Hepatitis Viruses

One report appeared that stated that the serum protein composition of hepatitis patients was not changed as compared to the normal.⁴³ In contrast to this, the results of other investigations showed that there was a hyperproteinemia present during the disease⁴⁴ and that a decrease in the albumin fraction occurred.⁴⁴⁻⁴⁸ Accompanying these changes there were increases in the serum globulins,^{48,49} particularly in the beta and gamma fractions.⁴⁴⁻⁴⁷ Antibody analytical methods were not available.

Canine Distemper and Infectious Canine Hepatitis

No changes were observed in the electrophoretic patterns of anticanine distemper sera.⁵⁰ The antibody activity was recovered in the serum globulins by salt fractionation methods.⁵¹ There appeared to be some relationship between complement-fixing antibody titers and the protective power of the sera.⁵¹ In anticanine distemper and anti-infectious canine hepatitis sera a pure gamma globulin was obtained by chemical fractionation. This material

contained only 50 per cent of all the antibody activity, but all of the antibody activity was recovered if both the beta and gamma globulins were extracted.⁵²

EPITHELIOTROPIC VIRUSES

Trachoma and Smallpox

Electrophoretic analyses of sera from trachoma patients in the acute and cicatricial stages have shown a decrease in the albumin/globulin ratio.⁵³

A hypoproteinemia and hyperglobulinemia resulting in a decrease in the albumin/globulin ratio were demonstrated in human smallpox infections. No immunological studies were conducted but the increase in globulins was thought to be a result of liver damage or to increase in antibody titer.⁵⁴

A study of the serum protein changes in cattle inoculated with smallpox vaccine was conducted to determine the alterations produced by various methods of inoculation. An insignificant decrease in the albumin and globulin fractions occurred when virus was administered by the subcutaneous and intracutaneous routes. Cutaneous inoculations of the viral vaccine produced more drastic changes than the two previous routes of inoculation. The alpha and beta globulins increased up to 100 per cent during the third week while gamma globulin increased more than 60 per cent during the second week. Albumin concentrations were depressed. The highest level of antibody was obtained after cutaneous administration of the virus.⁵⁵

Foot-and-Mouth Disease Virus

A considerable number of studies have been made of sera of cattle and guinea pigs experimentally infected with foot-and-mouth disease virus. Chemical analyses and electrophoretic separation of the serum proteins and subsequent studies on the distribution of antibodies and the various classes of antibodies have been conducted. For these reasons these studies are being divided into two sections.

(1) *Serum protein composition.* Significant differences in the serum proteins of cattle infected with foot-and-mouth disease virus were not found although, on occasion, some increase in the weight of gamma globulin was observed.⁵⁶ Vesicular stomatitis, a disease that simulates foot-and-mouth disease, apparently did not produce any changes in the serum proteins, either.⁵⁷ The minor changes that were sometimes observed were probably due to the general loss of condition due to malnutrition brought about by the animals not ingesting sufficient food because of the oral lesions and the pain involved. Antibody studies were not conducted in these two studies.^{56, 57}

Fractionation of the serum proteins by chemical methods showed that there were no significant differences in the amounts of gamma globulin precipitated from sera containing a low or high neutralizing antibody titer. This was demonstrated by the intradermolingual neutralization test in cattle. Both the euglobulin and pseudoglobulin of the same serum showed essentially the same neutralization index, especially in sera obtained earlier than the fourteenth day of convalescence. On the other hand a ratio of antibody content of euglobulin to antibody content of pseudoglobulin of 10:1 was encountered.

A similar variation in distribution of antibodies appeared to exist in the fractions obtained by electrophoresis, although the activity of gamma globulin was more marked. Direct evidence on antibody activity of other fractions was not obtained.⁵⁸

Active immunization of cattle with inactivated foot-and-mouth disease vaccine produced an increase in the total protein concentration within three weeks. A relationship was evident between the total serum protein level and the dosage of vaccine administered. In these instances the serum protein concentration rose to a greater level with a larger dose of vaccine. The rise in total serum protein was apparently due to an increase in the total globulin concentration. Many sera did not exhibit significant changes in protein composition. No relationship could be demonstrated between the increase in the gamma-globulin concentration and the degree of immunity.⁵⁹

Cattle infected with types A and O viruses showed increases in the serum gamma-globulin level. When hyperimmunized with type A virus the gamma globulin increased to a greater degree, although with type O virus the alpha globulin increased in concentration.⁶⁰ When guinea pigs were hyperimmunized electrophoretic analyses of sera showed increases in the alpha globulin. The amount of this fraction did not have any relationship to the level of complement-fixing antibodies, neither was the level of gamma globulin related to the complement-fixing antibodies.⁶⁰ Other studies of hyperimmune guinea pig serum have shown that no significant differences are evident in the serum protein composition.⁶¹

(2) *Distribution of various antibodies.* Attempts were made to study the various antibodies against foot-and-mouth disease virus in the postpartum milk and sera of infected heifers. It was found that postpartum milk contained specific neutralizing antibody activity at a level higher than that of serum from the same animal at seven days after inoculation but that at five days postpartum the neutralization indices of both serum and milk were similar. Neutralization tests were conducted in mice with virus cultivated in tissue culture. Complement-fixing antibodies of whey prepared from postpartum milk appeared to be lower than serum complement-fixing antibody titers. The whey antibodies were not detectable after ten days. Employing paper electrophoretic methods relatively high concentrations of whey proteins with the mobility of globulins were noted during the first five days postpartum. However, the concentration of these proteins decreased to an insignificant level by the tenth day.⁶²

The distribution of precipitating, neutralizing, and complement-fixing antibodies in foot-and-mouth disease has been studied extensively in the sera of cattle and guinea pigs.

The precipitating antibody present in 7-day convalescent bovine serum corresponds to a beta-type globulin. Subsequent immunoelectrophoretic analyses in agar showed that precipitin bands were obtained when 7- and 21-day convalescent sera were allowed to diffuse from adjacent cups toward the homologous virus. The bands, however, did not cross, indicating that the antibodies were identical.⁶³ Additional work on this phenomenon by the same laboratory showed that the mobility of the precipitating antibodies was

altered considerably during the convalescent period. Seven days after infection the precipitin band was produced in the beta-globulin area with the infective component of the virus. The antibody was also capable of neutralizing viral infectivity. After 14 days precipitation occurred in the gamma-globulin region. Two precipitating antibodies that reacted with both the infective and noninfective components of the virus were detectable. The 2 antibodies possessed the mobility of gamma globulins although the mobilities were not identical.⁶⁴

Chromatographic separation of the sera on diethylaminoethylcellulose columns further demonstrated the fact that the neutralizing activity and precipitating antibody of a serum were present in the same fraction. Antibodies in 14-day-or-more convalescent sera were eluted from the column with phosphate buffer at pH 7.6, whereas 7-day serum antibodies were retained in the column but were eluted with an acidic phosphate buffer. This buffer also eluted the beta globulin. The results of these 2 investigations^{63,64} justify the conclusion that there are intrinsic differences in the physical properties of neutralizing and precipitating antibodies present in the sera of cattle and guinea pigs at various periods after infection with foot-and-mouth disease virus.

Results of studies on the distribution and activities of the complement-fixing and viral-neutralizing antibodies in hyperimmune guinea pig sera have been reported.⁶¹ The investigation showed that a relationship did not exist between the levels of complement-fixing and viral-neutralizing antibodies. In cases where various pools of sera were prepared with identical complement-fixing antibody titers ranging from 1:10 to 1:320 only minor differences in neutralization indices were observed between each serum pool.

Separation of individual serum fractions was accomplished using continuous-flow paper curtain electrophoresis. Subsequent concentration of each fraction and immunologic and serologic assays showed that in instances where one serum protein component was separated on 2 adjoining drip points the albumin and leading alpha globulin were devoid of significant viral-neutralizing activity. The trailing alpha globulin was capable of reducing the virus LD₅₀ titer by at least four log units. The beta-globulin and both gamma-globulin fractions collected on adjoining drip points individually neutralized 6 logs of virus. In this particular instance the LD₅₀ titer of the virus control was 10⁻⁶. All neutralization tests were conducted in suckling mice. Additional studies consistently showed that all or nearly all of the viral infectivity was neutralized separately by the beta and gamma globulins.⁶¹ The usual neutralizing antibody activity of each serum fraction was as follows: albumin, 0.005 per cent; alpha globulin, 0.5 per cent; beta globulin, 100 per cent; and gamma globulin, 100 per cent.

When gamma globulin was successfully fractionated on adjoining drip points only the leading gamma-globulin fraction possessed complement-fixing antibody activity comparable to the activity of whole serum.

The differences observed on the relative activities of the neutralizing antibodies present in the same over-all serum fraction but separated as leading and trailing components on adjoining areas on the curtain may have been

due to slight differences in molecular structure that led to the observed differences in electrophoretic mobilities. This difference in molecular species was probably sufficient to explain the relative activities of these subfractions.

The lack of change in the electrophoretic composition of hyperimmune guinea pig serum as compared to normal serum may be explained by the fact that the immunization process and subsequent production of the various antibodies does not yield a sufficient weight of antibody to alter the amounts of the individual serum proteins. Another possibility, acting alone or in conjunction with the above, is that most of the normal globulin synthesis is directed to the production of antibody globulins without reflection in the amount of total globulins upon antigenic stimulus with foot-and-mouth disease virus.⁶¹

SUMMARY

The previously mentioned review has brought forth the facts that a single purified antiviral serum fraction is capable of possessing more than one serologic or immunologic manifestation. It is also evident that more than one purified serum fraction can possess similar serologic or immunologic activities. A plausible explanation may be brought forth in that there are characteristically minute differences in molecular orientation in the various antibodies associated with the serum fractions. These differences probably account for the dissimilarities in electrophoretic mobilities between the slow and fast components of one intact serum fraction and also for the different serologic and immunologic reactions that have been observed. It may also be that molecular structures are nearly similar between separate serum fractions that possess identical antibody activities although the inherent differences, possibly in terminal groupings, would be sufficient to account for the different electrophoretic mobilities.

Generally speaking, the explanation for the lack of significant changes in the plasma proteins of sera obtained from hosts inoculated with various viral preparations seems obvious. The weights of antibodies associated with a protein fraction are not sufficient to be evident as an increase in any one particular fraction when the components are separated electrophoretically or by chemical means even though the various antibody activities of the sera are high.

In cases where decreases in the albumin/globulin ratio have been observed it is more likely that the viral infections have produced a debilitating effect on the host and thus the capability of synthesizing albumin, for example, by the liver, is decreased. This would be reflected in turn as a higher proportion of globulin to albumin.

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BLOOD AND PLASMA VOLUME, TOTAL SERUM PROTEIN, AND ELECTROPHORETIC STUDIES IN HELMINTHIC DISEASES*

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The study of plasma proteins gained much impetus with the introduction of the Tiselius technique for electrophoresis. Early studies on large domestic animals usually ignored the possible influence that helminthic infection might have on the plasma proteins although conventionally raised animals of this group (horses, sheep, and cattle) harbor more than 20 different species of helminths and some degree of infection is usually present in all individuals.

As parasitologists began inquiry into the influence of helminthic infection on the plasma proteins, it soon became evident that marked changes could result in the electrophoretic patterns following infection of animals previously raised helminth-free.

Stauber (1954) has reviewed the literature concerning electrophoretic techniques as applied to parasitic diseases and has pointed out that with one exception (the malaria study in humans by Taylor *et al.*, 1949) all the reports considered did not take into account the influence of the pathological process on the total circulating plasma or serum proteins. Between 1937 (when moving-boundary electrophoresis was established as a precise and practical method of plasma protein separation by Tiselius) and the review of Stauber in 1954, apparently only three papers concerning the plasma proteins in helminth diseases exist. These are the work of Wright and Oliver-Gonzalez (1943) and Schwonzen (1951) with *Trichinella spiralis*, and Evans *et al.* (1955) with *Schistosoma mansoni*.

The introduction of the various paper techniques with their lower cost, ease of operation, and smaller sample requirements has resulted in increased interest in this area of study. Since Stauber's review (1954) a number of studies concerning the plasma proteins in helminthic diseases have been published. It is with these studies that the present paper will deal.

Animals and Animal Facilities

The procurement of small helminth-free laboratory animals has not been a difficult problem. However, in the case of horses, sheep, and cattle, economic as well as technical problems are encountered. Since nearly all animals become infected in their usual environment, it is necessary in the case of these large host species to obtain worm-free experimental animals. This has been done by taking the animals at birth and raising them in specially designed cages which provide a worm-free environment.

In the interest of economy of time and cost, various compromises have been made in the production of experimental animals in the case of the larger host species. Studies on superimposed infections or the comparison of "lightly"

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versus "heavily" infected hosts have been made. Obviously, the exact status of host susceptibility in these cases has been obscure, and certain reservations in the interpretation of the results have been necessary. On the other hand, one might argue the animals under the conditions necessary for a strict helminth-free environment do not represent normal individuals (caged and housed versus pasture conditions). It does, however, seem desirable to have a consistent base line from which to depart.

Nematodes

Nippostrongylus muris. Leland *et al.* (1955) compared the serum proteins in rats, injected at two-week intervals with increasing numbers of *N. muris* larvae, with uninfected litter mates. The larvae were repeatedly washed and were injected subcutaneously with antibiotics. In addition, the larvae were allowed to settle out of a portion of the inoculum and the larvae-free supernatant was injected into a third group of rats in an amount equal to the volume and number of injections given the infected group. This provided a control on the nonlarval portion of the inoculum and showed its influence on the serum proteins.

In the infected rats the total serum protein was slightly increased; the beta globulin distinctly increased as the number of larvae increased and diminished when the number of larvae injected was reduced, or when the larval injections were stopped; the gamma globulin showed no appreciable increase or decrease.

Serum patterns from rats that had received injections of the supernatant were essentially the same as those of the uninfected controls. This indicated that the material in the inoculum other than the larvae did not cause the observed serum protein changes.

Living larvae, saline extracts of living larvae, and ground larvae were used as antigens and exposed to immune sera. Antigen-antibody combination, as indicated by removal of all or part of an electrophoretic component after exposure of immune serum to the various antigens was not observed.

The lung weight when converted to per cent of total body weight was found to increase in the infected animal as compared with that of the control, and these increases were paralleled by the serum changes. This lung involvement caused by the migrating larvae was considered as the traumatic center that produced, at least for the most part, the observed serum changes.

It is interesting to note that Kruidenier and Katoh (1959) observed a distinct increase in the beta-gamma globulin serum fraction of rats and cats infected with the lung fluke *Paragonimus kellicotti*. Furthermore, Kraut (1956) observed among other changes a significant beta-globulin increase in rats infected with the larval cestode *Cysticercus fasciolaris*.

Trichostrongylus axei. This is a minute stomach worm of horses, sheep, cattle, and other hosts. Leland *et al.* (1958, 1959b) made pre- and postinfection measurements as well as parallel measurements on helminth-free controls. Calves were maintained from birth through the experimental period under conditions where unintentional acquisition of helminths was eliminated.

The most consistent and prominent serum protein alterations were (1) an extensive and prolonged hypoproteinemia as evidenced in grams/100 ml. and

total grams of circulating protein, and (2) a hypoalbuminemia with an increase or maintenance of the alpha-2-globulin level and in general a decrease of the other globulins all of which were manifested in per cent composition, grams/100 ml., and grams of total circulating component (FIGURES 1 and 2).

The sequence of events with respect to blood and plasma volumes (Evans blue method) in infected animals appears to be a development of polycythemic hypovolemia that, if the animal survived, developed into an oligocythemic hypovolemia. At a point in the transition from the former to the latter condition, a state of simple hypovolemia thus existed.

In experiments identical in design with the calf study and using the same strain of the worm, Leland *et al.* (1959a and 1960b) observed in lambs the following serum protein alterations: (1) hypoproteinemia as evidenced in grams per 100 ml. and total grams of circulating protein, and (2) a hypoalbuminemia with an increase in gamma globulin (FIGURES 3 and 4). As in the calves, blood and plasma volume alterations in infected lambs were the development of polycythemic hypovolemia which, if the animal survived, developed into an oligocythemic hypovolemia (FIGURES 5 and 6).

Quantitative alterations in any plasma protein constituent should reflect an alteration in rate of synthesis, rate of destruction,* and/or external loss of that constituent.

The assault on the physiological processes of the host by severe helminthic infection is accompanied in general by such indirect effects as malnutrition and negative water balance initiated by the worm. Thus the direct effects of the parasite are supplemented and sometimes overshadowed by the indirect effects.

Mechanisms that lead to a reduction of serum proteins are: (1) diminished formation as the result of (a) deficient protein intake, (b) damaged liver, an important formation site of plasma proteins, and (2) excessive loss. Lesser reductions have been reported in chronic infection, instances of impaired absorption of protein constituents, chronic diarrhea, and open suppurative draining wounds.

Feed consumption and corresponding protein intake were drastically reduced in lambs and calves severely affected by *T. axei* infection. Factors in addition to deficient protein intake, however, may have contributed to the hypoproteinemia. A toxin, perhaps, in the form of a metabolite of the worm, has been postulated as the actual cause of the gastric disturbance, inflammation, weakness, emaciation, and anemia associated with *T. axei*. The existence or the influence of such a toxin on the formation sites (liver) of the plasma proteins has not been established. Loss of protein via the kidney in the case of the infected calves was not observed.

Another possible means by which plasma protein is lost in the severely affected animals may be through the alimentary tract via the abomasal lesions. Since polycythemia occurs at about the time the plasma proteins decrease, the loss of plasma protein via the abomasal lesions would have to be effected

* As this paper goes to press, Smithers and Walker (1961) have reported that the fall in albumin in monkeys infected with *Schistosoma mansoni* was due to a sudden increase in albumin catabolism incompletely compensated by an increase in anabolism.

without the loss of blood cells. This situation is not unique since loss of plasma alone in burns occurs both from the surface and into the tissues surrounding the burn. In extensive burns as much as 70 per cent of the total plasma volume may be lost from the circulation within a few hours. In

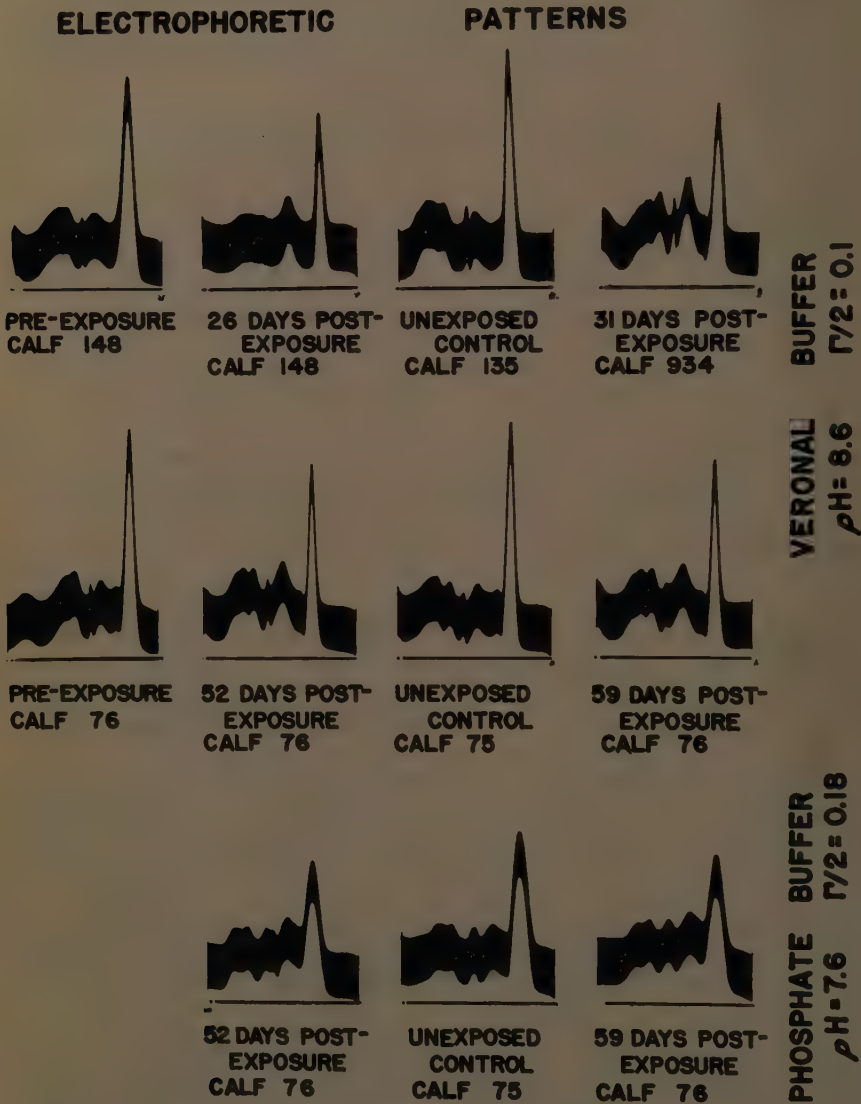


FIGURE 1. Sample electrophoretic patterns (descending limb) of calf serum from pre- and postinfection (*T. axei*) and the uninfected control calves for the corresponding period. The protein concentration of the solution analyzed was 2 per cent in all cases except calves 148 and 135, where one part serum/two parts buffer dilution was made. Potential gradients of 7.9 volts/cm. for 2 hours (veronal) and 7.25 volts/cm. for 2½ hours (phosphate) were maintained. Reproduced by permission of *Experimental Parasitology*.

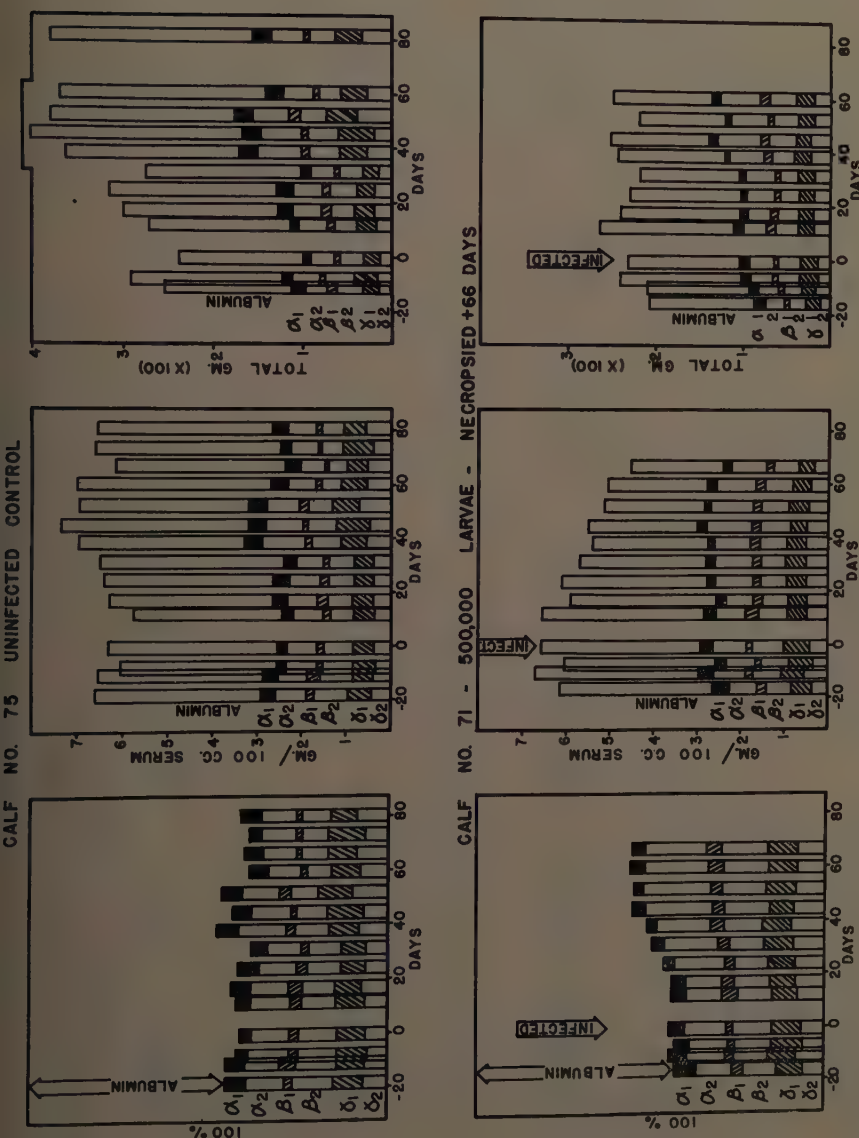


FIGURE 2. Electrophoretic serum fractionation in calves uninfected and infected with *T. axei*. Ordinates are: 100 per cent = components expressed as per cent of total serum protein (per cent composition); gm./100 cc. = protein components expressed in gm./100 cc. serum (concentration); total gm. = components expressed as total grams of circulating serum protein per animal. Reproduced by permission of *Experimental Parasitology*.

ELECTROPHORETIC PATTERNS OF LAMB SERA (pH 8.6)

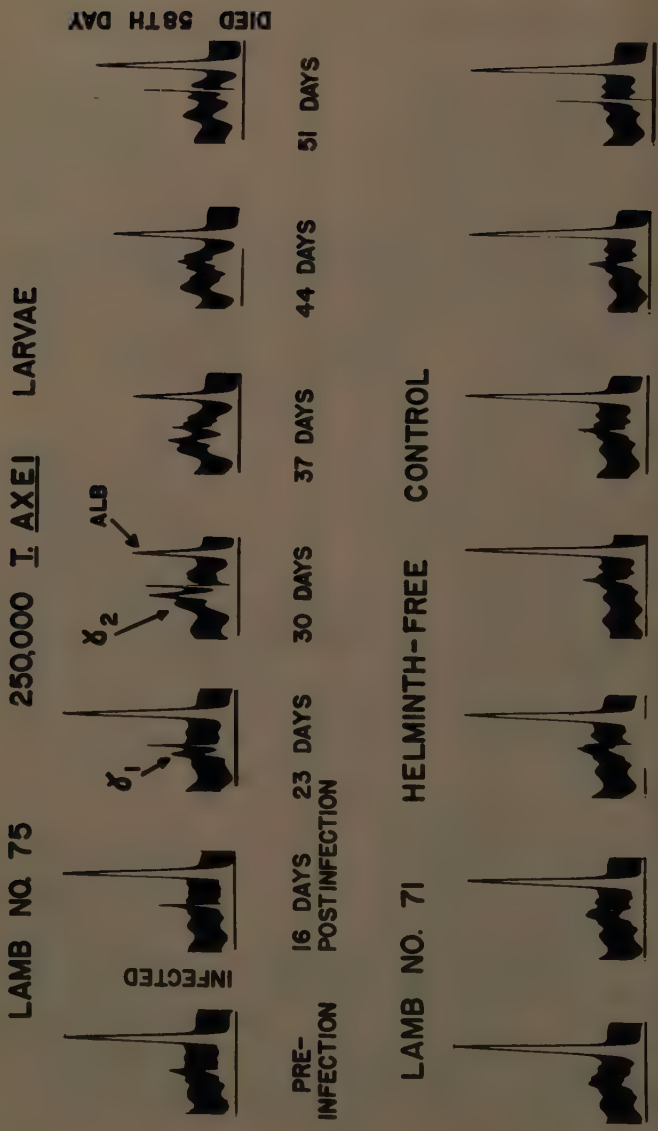


FIGURE 3. Sample electrophoretic patterns (descending limb) of serum from an infected lamb (*T. axei*) and an uninfected control lamb for the corresponding periods. Analyses were made at a protein concentration of 2 per cent, in veronal buffer at pH 8.6 with a potential gradient of 7.9 volts per centimeter for 2 hours. Reproduced by permission of the *American Journal of Veterinary Research*.

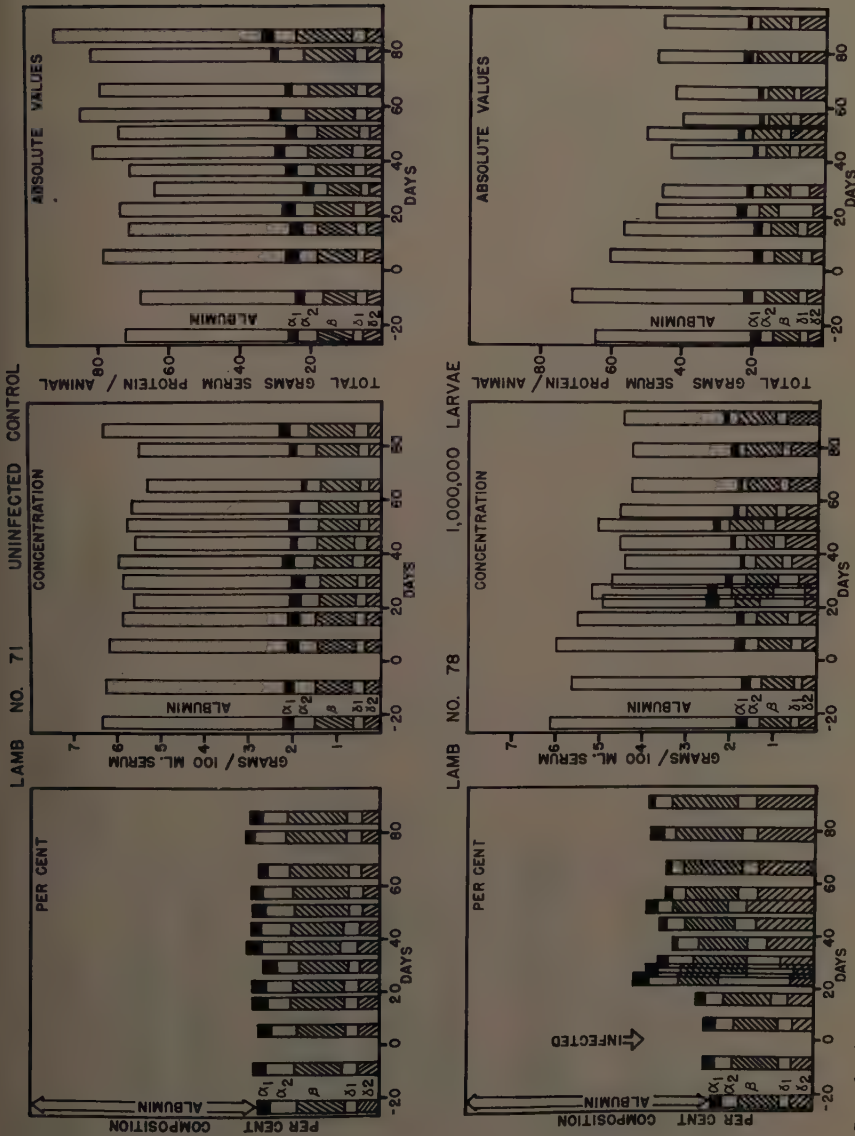


FIGURE 4. Sample data from electrophoretic fractionation of serum from lambs uninfected or infected with *T. axei*. Reproduced by permission of the American Journal of Veterinary Research.

addition, calves experimentally burned show a decrease in albumin and an increase in alpha globulin of the serum (Perlman *et al.*, 1943).

Elsewhere in these pages, H. Tarver discusses a normal "leakage" and breakdown of serum albumin in the rats' intestinal tract. In pathological conditions, loss of protein from the gastrointestinal tract appears to be increased.

A condition in humans known as giant hypertrophy of the gastric mucosa,

BLOOD AND PLASMA VOLUMES LAMB NO. 77A 750,000 LARVAE

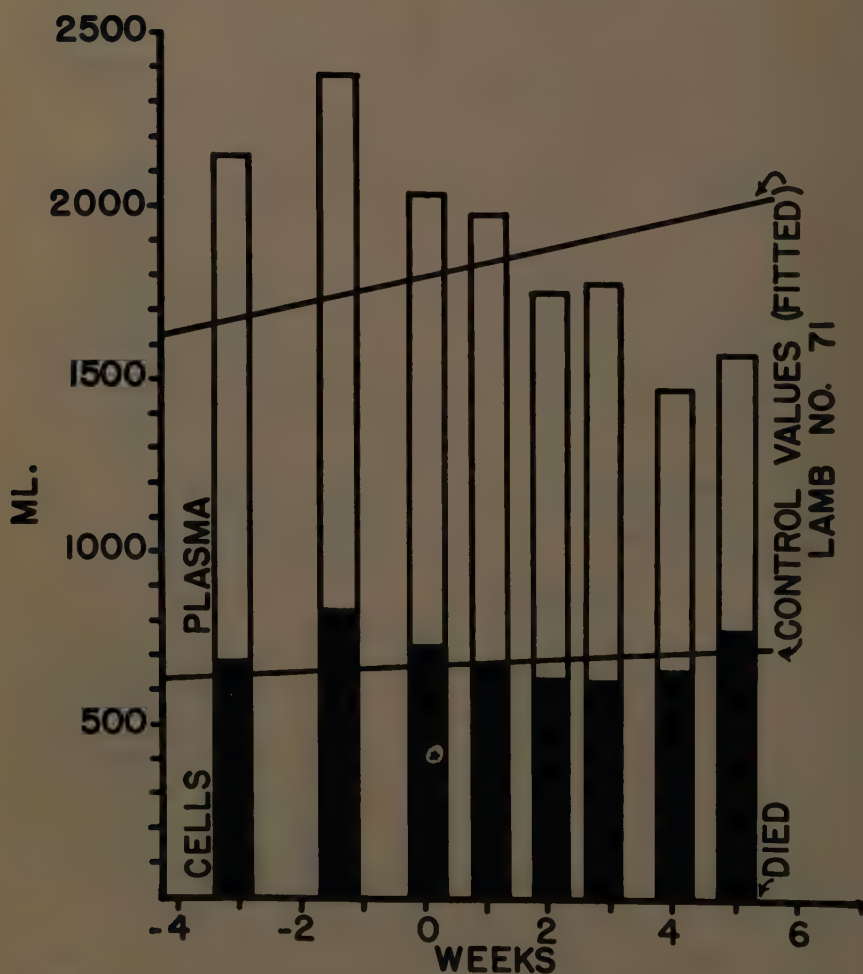


FIGURE 5. The influence of infection with *T. axei* on the blood and plasma volumes of a fatal case. Reproduced by permission of the *American Journal of Veterinary Research*.

LAMB NO. 78 1,000,000 LARVAE

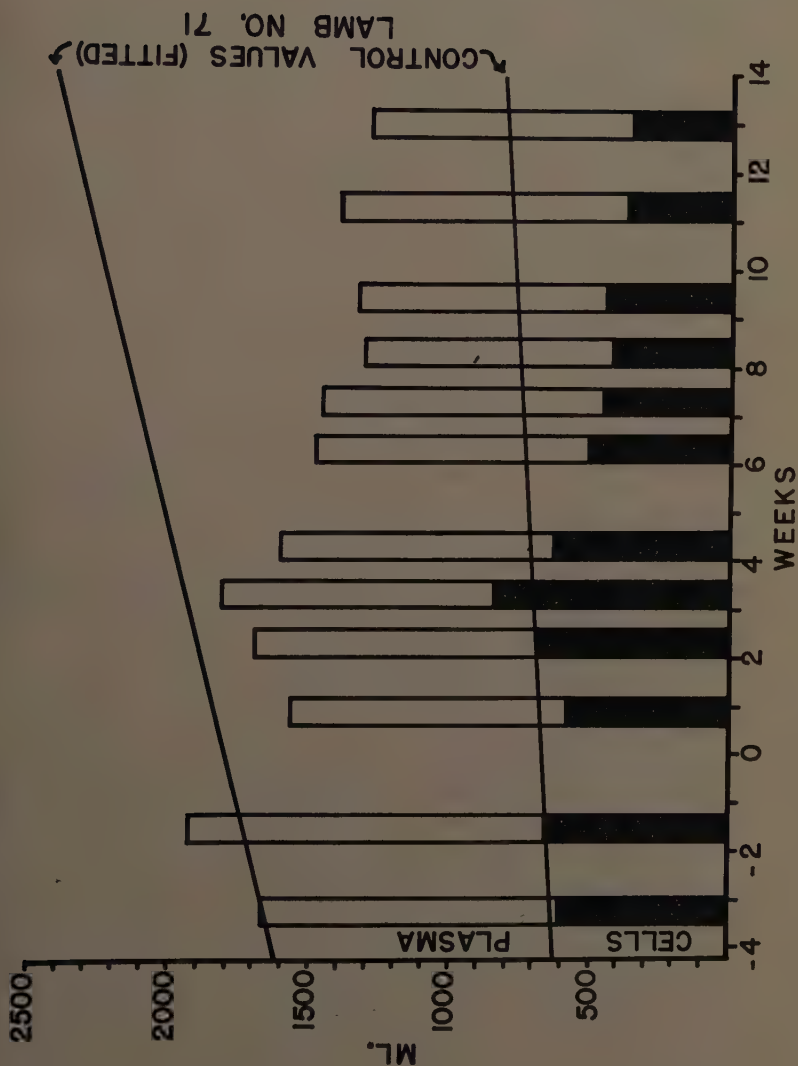


FIGURE 6. The sequence of events with respect to blood and plasma volumes in a lamb that survived 13 weeks after experimental infection with 1,000,000 larvae. Reproduced by permission of the *American Journal of Veterinary Research*.

giant hypertrophic gastritis, and Menetrier's disease occurs as a relatively uncommon lesion of the stomach. Hypoproteinemia is sometimes associated with the disease. The mechanism of the lowered serum protein has been ascribed to loss of serum through the hypertrophied gastric glands or to an altered stomach function which normally facilitates protein absorption. Using I^{131} -labeled albumin, it has been shown that there is a loss of protein into the gastrointestinal tract from the hypersecretion of plasma proteins in the gastric juice. Although its degradation products are absorbed, the albumin is lost to the circulation and tissues since the body is unable to convert these amino acids into albumin at a sufficient rate to compensate for the loss of protein (Citrin *et al.*, 1957).

Noteworthy in the case of *T. axei* infection is the fact that with the existing hypoalbuminemia, conditions are optimum for generalized edema. The absence of generalized edema is apparently accountable to the general state of dehydration due to the reduced fluid intake and/or excessive fluid loss in diarrhea.

The results of the electrophoretic serum fractionation provide an interesting variation in that distinctly different patterns were observed in lambs and calves experimentally infected with the same strain of *T. axei*.

The situation as seen in the lambs, that is, the drastic reduction in albumin and minor changes of alpha and beta globulin, could tentatively be explained as a result of the liver involvement since this organ is the probable formation site of serum albumin and of alpha and beta globulins. The accompanying increases in the gamma globulins could be the result of (1) an alteration in the rate of synthesis, (2) antibody formation against (a) the worm, (b) invading microorganisms that have gained entrance via the abomasal lesions, or (c) the metabolic products of the worms or microorganisms, and (3) a compensatory reaction to restore serum osmotic pressure that would be greatly reduced as a result of the low albumin concentration.

Although no statement of finality can be made at this stage with regard to the mechanism involved in the observed serum protein changes, a few points of interest have evolved. Liver involvement cannot be accepted unequivocally as the sole cause for the above-described reductions in albumin and alpha globulin in sheep since, in calves where no perceptible liver damage was seen, the alpha globulin was greatly increased but the albumin drastically reduced. One might postulate in the case of the calves that any damage to the liver, although not visible, was sufficient to reduce the production of albumin but sufficient to stimulate the over-production of alpha globulin, and that when the damage becomes extensive the picture as seen in sheep would predominate. The possible influence on the liver of malnutrition, a toxin liberated by the worm, or toxic substances released by the extensive tissue destruction may also be contributing factors.

Although the autogenous production of gamma globulin begins shortly after birth in the case of calves (Pierce, 1955), the machinery necessary for extensive gamma-globulin production may be inadequately developed or unreliable in the young bovine. In addition, the debilitating effects of *T. axei* may selectively influence protein synthesis. Evidence exists in rats (Miller and Bale,

1954) that indicates the alpha globulins are normally produced and turned over at a more rapid rate than any of the other protein fractions.

From experiments just completed, from which quantitative information is not yet available, older pastured calves with superimposed experimental infections first produced a marked increase in alpha-2 globulin, which later decreased. As the decrease in alpha-2 globulin occurred a marked increase in the gamma globulin resulted.

Thus the alpha globulin, being more readily produced in young calves, may act in a compensatory role to restore reduced osmotic pressure resulting from the hypoalbuminemia. Both albumin and presumably alpha globulin (fetuin) make the major contribution to plasma osmotic pressure in the newborn calf (Pierce, 1955). The increase in alpha globulin may be a temporary situation allowing time for the slower and perhaps more difficult production of gamma globulin by the young bovine.

An increase in alpha globulin appears to take place when there is present any considerable inflammation or tissue destruction, irrespective of its cause (Shedlovsky and Scudder, 1942). In this respect, the increased alpha globulin in calves is in keeping with the extensive pathological manifestations produced by *T. axei*.

The alpha globulin increase may be in response to cellular reconstruction requirements. Kent and Gey (1957) have shown that alpha globulin disappears from the medium in which malignant cells are grown and Puck (1960) has associated increased *in vitro* cell multiplication and glass spreading properties with fetuin.

Finally, to what extent the alpha globulin increases (as determined by the moving boundary method) are due to increased glycoprotein has not yet been determined. As pointed out elsewhere in these pages by Heiskell *et al.*, alpha glycoproteins are increased in infectious and inflammatory diseases.

Possible factors contributing to the reduced plasma volume were (1) negative water balance brought about by reduced intake and increased loss associated with the diarrhea and loss via the abomasal lesions; (2) reduced plasma protein brought about by a reduced rate of synthesis and/or loss via the abomasal lesions, and (3) reduced protein intake. The reduction in cellular volume observed toward the end of the observations period may be the result of (1) prolonged anhydremia, (2) the anemia associated with long-standing infections where increased blood destruction may not be the causative factor but, instead, a disturbance in iron metabolism accompanied by a reduced plasma iron and iron-binding protein; (3) a reduction in the erythrocyte life span of sufficient magnitude that the bone marrow cannot compensate; (4) a specific nutritional deficiency; (5) autoimmune anti-red blood cell antibody formation; and (6) toxins resulting from *T. axei* infection which interfere with the blood-forming cells.

Ostertagia ostertagi. As regards this medium stomach worm of cattle: in three calves showing clinical symptoms of parasitism and reduction in weight gain following 25,000 to 50,000 larvae, Anderson *et al.* (1960) observed a marked albumin decrease and an increase in one or more of the globulins.

In 4 calves each inoculated with about 300,000 *O. ostertagi* larvae, the re-

sults were similar except that the controls made abnormally low weight gains, and the serum proteins underwent changes similar to those of the infected animals. The calves of this experiment had been exposed to severe winter weather during much of this experiment.

No consistent difference between infected and control animals was found in total protein (determined weekly) or in plasma volume (determined biweekly).

Haemonchus placei. In 7 calves inoculated with 12,500 to 165,000 larvae (antelope strain) no marked symptoms of parasitism, reduction in weight gain, or changes in serum proteins were observed by Anderson *et al.* (1960).

No consistent difference between infected and control animals was found in total protein (determined weekly) or in plasma volume (determined biweekly).

Dictyocaulus viviparus (lungworm). Weber (1957) studied the serum proteins of cattle exposed and re-exposed to the lungworm in an attempt to correlate the increase in gamma globulin with antibody formation and eosinophilia. Gamma globulin increased beginning about one week after infection, attained a maximum four weeks later, persisted for different periods according to the number of exposures, and then gradually returned to normal. Although no direct quantitative relation between gamma globulin, antibody titers, and eosinophilia was demonstrated, increases in gamma globulin and percentages of eosinophils appeared to be associated with antibody formation and titer in the serum of cattle infected with lungworms.

Djafar *et al.* (1960) observed in calves that survived experimental infection, significant increases in gamma globulin that began shortly after oral inoculation of the previously helminth-free animals. A peak in gamma globulin was reached on postinoculation day 35.

In calves that succumbed there was a moderate increase in gamma globulin that was delayed until after postinoculation day 14.

Strongyloides papillosus. Turner (1957, 1959) compared the serum proteins of a helminth-free lamb with lambs exposed cutaneously to 150,000 infective larvae of *S. papillosus*. The total serum protein varied within normal limits in all animals throughout the experimental period (6.0 ± 0.5 gm. per 100 ml. of serum), although minor variations occurred between individuals. The beta and gamma globulins increased during the infection. The highest concentration of beta globulin appeared the third week after infection and subsequently decreased to lower levels, whereas the gamma globulin increased gradually, reaching a maximum concentration the sixth week after infection. These increases in beta and gamma globulin are attributed to "antibody" formation. Turner, however, properly pointed out that the evidence relating the globulin increases to antibody formation was indirect. Thus this interpretation can be acceptable only with some reservation since it was not shown that the observed beta and gamma globulin increases were largely due to antibody globulin and not the result of a nonspecific type of response initiated by trauma produced by *S. papillosus*. The *in vitro* absorption of an electrophoretic serum component following contact with some form or preparation of the worm would be one way of establishing the antibody nature of the observed increases.

Mixed gastrointestinal nematode infections. Shumard and Eveleth (1955) and Shumard *et al.* (1957) observed in four young lambs experimentally infected with equal numbers of *Haemonchus contortus*, *Nematodirus spathiger*,

and *Trichostrongylus colubriformis* larvae that all animals showed a depression in the total serum protein and the albumin:globulin ratio (by methanol precipitation).

Kuttler and Marble (1960) recorded the serum protein changes in lambs with naturally acquired clinical, moderate, and light (controls) infections of mixed nematodes. In addition, the serum proteins of lightly parasitized animals with anemia induced by periodic bleeding, were measured to determine whether the anemia often associated with moderate to clinical parasitism was actually responsible for the serum protein changes.

Significant changes in serum protein occurred with moderate and clinical parasitism. In clinically parasitized lambs, the total serum protein was decreased; albumin fraction was decreased; and the alpha-1, alpha-2, beta, and gamma-globulin fractions were increased. The net difference between the clinically parasitized lambs and the lightly parasitized lambs with induced anemia was a significant increase in the alpha-2 globulin and a significant decrease in the albumin. The authors concluded that changes do occur in serum protein fractions independent of changes associated with anemia.

Turner and Wilson (1960) studied the effects of 3 different exposures of parasitisms on the serum proteins of lambs over a 10-month period. The average gamma globulin percentages were relatively constant in lambs of a helminth-free group and a barn-restricted group whereas, in a pasture group, the gamma globulin rose steadily after the fourth month to about 3 times that of the helminth-free and barn groups at the termination of the study. The average albumin-to-globulin ratio declined in the heavily parasitized pasture group but increased steadily in the other 2 groups.

Ross *et al.* (1959) described a genetic line of zebu cattle that exhibited resistance to haemonchosis. The resistant cattle tended to show an increase in the faster-moving gamma-1 globulin, while the low-resistant cattle showed the increase in the slower moving gamma-2-globulin fraction. The differences were not considered significant.

The progeny of the resistant group (Ross *et al.*, 1960a) when infected with gastrointestinal helminths of the order Strongyloidea, showed comparatively low fecal egg counts and high complement-fixing antibodies. Comparison was made with the susceptible line of cattle.

An increase in the gamma-1-globulin fraction was associated with an increase in complement fixation index ratio in the resistant group. In the susceptible group the gamma-1-globulin level remained relatively static and lower, and the complement fixation index ratio remained low.

The gamma-2-globulin fraction appeared to follow the course of the worm infestation as reflected by worm egg counts in both progeny groups.

Ross and Armour (1960b) stated the serum albumin level and packed cell volume percentages were useful measures of pathogenicity when considered in conjunction with a series of differential worm egg counts.

Cestodes

Cysticercus fasciolaris. This is the larval form of a common tapeworm of cats, *Taenia taeniaeformis*; rats or mice serve as the intermediate host. Kraut (1956) analyzed electrophoretically the sera of rats experimentally infected

with the larval cestode *C. fasciolaris*. Similar analyses were made on sera of rats artificially immunized with larval worm material. Significant quantitative increases in heavily infected rats occurred in the alpha-2, beta, and gamma globulins with a decrease in albumin. In rats containing fewer larvae than the heavily infected rats, only the beta globulin and albumin differed significantly from the uninfected controls. No significant change in total serum protein was observed.

The rats, artificially immunized with ground cysticerci in order to circumvent serum changes resulting from liver damage, did not show the serum changes although immunity was produced.

Electrophoretic analysis of serum before and after exposure to various worm material *in vitro* showed no significant differences.

The observed serum changes in the infected animals are interpreted as a reflection of the extent of liver involvement.

Trematoda

Schistosoma mansoni. A comparative ionographic study of the sera of hamsters, mice, and albino rats experimentally infected with cercariae of this human blood fluke was made by Evans *et al.* (1955) and by Evans and Stirewalt (1958). In terms of (1) the proportion of parasites which matured, (2) the protective tissue reaction against the parasites or their eggs, and (3) the death rate versus infection level, the hamster, mouse, and the rat, respectively, show a reduction in host susceptibility to the parasite.

In the hamster the gamma and beta-2 globulins of the infected animals rose steadily during the 6 months experimental period with the beta-2 rise evident at 8 weeks and the gamma rise evident at 16 weeks. In terms of percentage composition the albumin decreased, whereas the albumin concentration (grams per liter) remained remarkably constant. A steady rise in total protein was totally accounted for by the gamma and beta-2 globulin increases.

With increasing age of the control animal, a steady decrease of alpha-2 globulin to half its original volume was noted while the infected hamster maintained a constant level of this component. Thus it must be concluded that the alpha-2 globulin also steadily increased as a result of infection. This would be in keeping with the general concept that alpha globulin increases when there is present any considerable inflammation or tissue destruction irrespective of its cause.

The picture in the mouse did not differ greatly from the hamster. In both hosts there was a sharp initial rise in the gamma-beta-2 globulin experimental: control ratio which was followed by a decrease in mice but not in hamsters. This longer maintenance of the increased gamma-beta-2 components in the hamster may be a manifestation of the greater susceptibility of the hamster over the mouse.

Serum protein changes in the mouse were first observed at a point in the infection (at the end of the 12th week) where considerable damage had been sustained by deposition of eggs in the liver.

In rats there was almost a complete lack of response to the infection. The

beta globulin appeared to be increased about 29 per cent over the control values at 26 weeks after infection, but these authors considered this merely to be a reflection of the variability of this component.

A serological characteristic of human schistosomes is the formation of an enveloping membrane (CHR) around cercaria suspended in the sera of infected hosts. The reaction is not species-specific but does not occur in sera of uninfected hosts. In addition, agglutination of cercariae, cercaricidal activity, and precipitate formation have been reported upon exposure of cercariae to various sera.

These authors (Evans *et al.*, 1955) performed the cercaricidal and CHR tests in electrophoretically (Tiselius) separated fractions of sera from uninfected and infected mice. Cercaricidal and pericercarial membrane activity were largely confined to the descending limb with the cercaricidal property moving with the slower and the pericercarial membrane-forming property moving with the faster components. It was concluded that the "gamma" sample of infected mouse serum contained the factors responsible for both cercaricidal activity and pericercarial envelope-formation and that the third or "alpha" sample introduced a cercaricidal inhibitor which thus made it possible for the cercariae to form envelopes. Envelopes were found, therefore, only in those infected serum samples that contained both gamma and alpha globulins or at least with components having mobilities similar to these fractions.

Their studies (Evans and Stirewalt, 1959) were extended and further refined by use of the continuous flow electrochromatographic technique. Sera of untreated human cases of *S. mansoni* were fractionated into 6 major components and a total of 28 subfractions. Each subfraction was tested for CHR and cercarial agglutinating activity. Both activities were localized in a single subfraction that comprised about 0.6 per cent of the total protein fraction. The active principle isolated was soluble in triple-distilled water and was an electrophoretically homogenous, fast-moving portion of the gamma-1 globulin (T-globulin) in 0.1 *M* veronal buffer, pH 8.6. Quantitative ultracentrifugal analysis showed the fraction to be polydisperse, having two minor comparatively fast sedimenting components and a major slow sedimenting component. CHR and cercarial agglutinating activity were isolated in the major component. Amino acid composition was found to be somewhat different from that given for "normal" human gamma globulin.

Cancio *et al.* (1959) fractionated sera from humans infected with *S. mansoni* and from noninfected controls by continuous flow paper curtain electrophoresis. The circumoval-precipitin test was performed on all fractions obtained. It was observed that the circumoval precipitin antibody present in the blood sera of individuals infected with *S. mansoni* was located in the gamma-1 globulin fraction. The other fractions that is, albumin, alpha-1, alpha-2, beta, and gamma-2 globulins were negative for circumoval precipitins, as were all fractions of sera from the noninfected human controls.

In 10 human patients with *S. mansoni* infection accompanied by hepatosplenomegaly, Fiorillo (1954) observed a marked increase in relative beta-gamma components.

Studying the effects of malnutrition on schistosomiasis, DeWitt (1957) rendered mice deficient in Factor 3, vitamin E, and cystine with a *Torula* yeast diet. When uninfected mice on the *Torula* yeast diet for 68 days were compared with mice on a complete diet, the former showed 14 per cent decrease in total protein, 23 per cent decrease in albumin, 20 per cent increase in beta globulin, 50 per cent decrease in gamma globulin, and 26 per cent decrease in A/G ratio. The alpha globulin level remained unaltered. *S. mansoni* infections in these deficient animals produced little or no further alteration in the serum protein levels. In mice on a full diet, *S. mansoni* infections resulted in marked changes in serum proteins. There was an increase in total protein which reflected an increase in the globulin components. The greatest change was in the gamma globulin, which rose from 0.4 gm. per cent to 1.5 gm. per cent. The A/G ratio of the infected mice averaged 0.7 as compared with 1.5 for uninfected controls. The failure of *S. mansoni* to produce alterations in serum protein levels of the deficient animals was interpreted as either an incapacity of the deficient animals to respond to the infection or to the inability of the stimuli produced by the underdeveloped worms to elicit a response.

Schistosoma japonicum. In infected rabbits beginning with oviposition a marked increase in total protein, a sudden relative (per cent composition) decrease in albumin, and an increase in gamma globulin occurs. In infected humans a significant decrease in albumin and increases in total protein, alpha-1 and gamma globulins were reported by Sadun and Walton (1958).

Fasciola hepatica. Using paper electrophoresis, Kóna (1957a) examined the serum of 37 sheep, 1-2 years old, which had anemia due to heavy infection from this common liver fluke. A marked increase in the relative gamma globulin, a slight increase in the alpha globulins, and a reduction in albumin was observed. Samples of peritoneal and pericardial fluid were also examined and compared with serum samples. The protein fractions in the serum and peritoneal fluid were about the same, but in the pericardial fluid a slight increase in albumin and a decrease in gamma globulin was recorded.

Fasciola gigantica. Weinbren and Coyle (1960) recorded a significant increase in "gamma" globulin with a fall in albumin in infected zebu cattle as compared with a control group. These findings were consistent with the considerable degree of irreversible liver damage that was present.

Paragonimus kellicotti. In rats and cats a distinct increase in the beta-gamma globulin accompanied infection of both species with this lung fluke. The increase appeared to be due primarily to an increase in gamma globulin. Little or no change appeared to occur in the actual (concentration) albumin and alpha-globulin fractions although relative decreases were apparent (Kruidenier and Katoh, 1959).

Conclusions

The fund of information concerning the influence various helminthic diseases exert on the plasma proteins can be classified as being in the early stages of development. At the present stage much of the limited effort has been directed toward establishing the type and extent of plasma protein changes that occur as a result of helminthic infection. Even less attention has been

paid to the changes in the total circulating plasma or serum proteins that require determination of plasma volume in addition to protein concentration and electrophoretic fractionation of the plasma or serum proteins. Where plasma protein changes have occurred the precise mechanisms responsible are largely conjectural.

Alterations in blood and plasma volume at least in the case of calves and sheep following infection with the stomachworm *Trichostrongylus axei* (Leland *et al.*, 1958, 1959a, 1959b, 1960b) were the development of polycythemic hypovolemia which, if the animal survived, developed into oligocythemic hypovolemia. At a point in the transition from the former to the latter condition, a state of simple hypovolemia thus exists.

In the only other study that considered blood and plasma volumes, Andersen *et al.* (1960) in a preliminary report observed no difference between calves infected with *Ostertagia ostertagi* or *Haemonchus placei* and their uninfected controls. These negative findings may be due to a low level of infection as indicated by the establishment of a small number of worms.

Depending on the host and parasite considered, a slight increase, no noticeable change, or a drastic reduction in total serum protein values has been reported. Slight increases in total serum protein are sometimes accounted for by increases in one or more of the various globulins. In other instances where no change in total serum protein occurs relative changes in the concentration of serum components takes place. In the case of drastic reductions in total serum protein, the albumin and several of the globulins may be reduced. The total serum protein (concentration) reduction, at least in the case of *T. axei* has been indicative of total circulating serum protein reduction.

Following helminthic infection major changes in the electrophoretically determined serum proteins can occur. Depending on the host and parasite considered, these changes have been manifested singly or in various combinations in all of the major components. There is no diagnostically specific picture for a particular helminthic infection or for helminthic infections in general. Where serum changes have occurred as a result of experimental helminthic infection the general picture most often has been a reduction in albumin accompanied by an increase in one or more of the globulins.

Although specific antibodies against helminths or their metabolic products have been shown to be present in the serum of exposed animals, there is apparently no evidence at present that would indicate that the marked increases observed in the globulin of infected animals are wholly or largely in response to the antigenic nature of the worm. Most serological techniques appear to have quantitatively accounted for only minor portions of the increased globulin. It has been shown, for example (Evans and Stirewalt, 1959), that certain antibodies of schistosomes are restricted to a single subfraction of the gamma globulin comprising about 0.6 per cent of the total protein fraction. Following inoculation various serological manifestations of antibody formation have been correlated with increases in various globulins. This evidence relating antibody to the marked increases in globulin is at present indirect. In many cases the globulin changes can also be correlated with the state of severity of the infection as can similar globulin changes be produced with physical and chemical injury

to the host. Extensive increases in globulin components induced by helminthic infection have not always been associated with the development of immunity. Furthermore, in animals with low levels of infection or in those exhibiting a spontaneous loss of parasites, there may be no quantitative increase in the globulin.

Evidence in favor of the nonspecific nature of the quantitative globulin alterations in helminthic diseases, although not voluminous, appears more convincing. The use of artificial immunization with ground worm material (Kraut, 1956) has shown that immunity could be produced without the marked increases in globulins that normally occurred with infection where liver damage was present.

In experimental infections using increments of infective larvae, clinical manifestations are generally proportional to the number of larvae inoculated (Leland *et al.*, 1959c, 1960a). Furthermore, serum-protein changes in *T. axei* (Leland *et al.*, 1959b, 1960b) appear to be proportional to symptomatology that in turn was generally the result of the number of infective larvae administered. It should be noted that in the majority of helminth species multiplication of the parasite within the host does not occur, and this makes quantification of the infection more predictable than in the case of bacterial and viral diseases. Thus the utilization of experimental helminthic infections should provide an additional approach to the basic study of traumatic and inflammatory conditions in general, since the host can be subjected to graded levels of exposure to the parasite.

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SERUM GLYCOPROTEINS IN INFECTIOUS AND INFLAMMATORY DISEASES*

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The serum glycoproteins have been reported to be increased in tuberculosis,¹ neoplastic disease,² rheumatoid arthritis,³ psoriasis,⁴ eczema,⁴ systemic lupus erythematosus,³ diabetes,⁶ pemphigus,⁷ dermatitis herpetiformis,⁷ and allergic diseases.⁸ Although the reported abnormalities of glycoproteins have been frequently confirmed for most of these diseases, they have not been widely used for diagnostic and prognostic purposes in clinical medicine. This may be attributed partly to an assumed nonspecificity of these abnormalities as well as to lack of information concerning the pathologic significance of these changes.

The purpose of this report is to compare quantitative and electrophoretic variations of certain glycoproteins in clinically discrete diagnostic categories and to compare these glycoprotein variations to those of C-reactive protein, a nonspecific measure of the inflammatory response widely used in clinical medicine.

Material and Methods

All patients were evaluated by physicians with a particular interest in the diagnostic category concerned. Serum was stored at -10°C . and thawed not more than 24 hours before testing. Total serum glycoproteins (TSGp) were determined by Weimer and Moshin's modification⁹ of the procedure of Lustig and Langer.¹⁰ Seromucoid protein (Smc) was determined on the isolated fraction by the method of Lowry and his associates.¹² Seromucoid hexose (Smc Hx) was determined by a modification⁹ of the procedure of Winzler and associates.¹¹ Serum electrophoretic patterns were determined with the Spinco system,¹³ using the Periodic-acid-Schiff stain and the Model RB Analytrol for quantitation. C-reactive protein levels were determined by a quantitative gel-diffusion technique.¹⁴

The individual electrophoretic patterns were categorized according to their profile when plotted on standardized scales (FIGURE 1). These scales were constructed so that a central horizontal line intersected the scale for each electrophoretic fraction at the normal mean value. Above and below the line representing the normal mean value were placed horizontal broken lines that intersected the scales at one, two, and three standard deviations from the normal mean. Beyond three standard deviations, the values were arbitrarily scaled to include extreme values. The values on the left side of each scale represent the percentage of total serum glycoproteins in that electrophoretic fraction; and the values on the right side of the scale, the absolute content computed from the total serum glycoprotein value and the percentage in the

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HEALTHY ADULTS

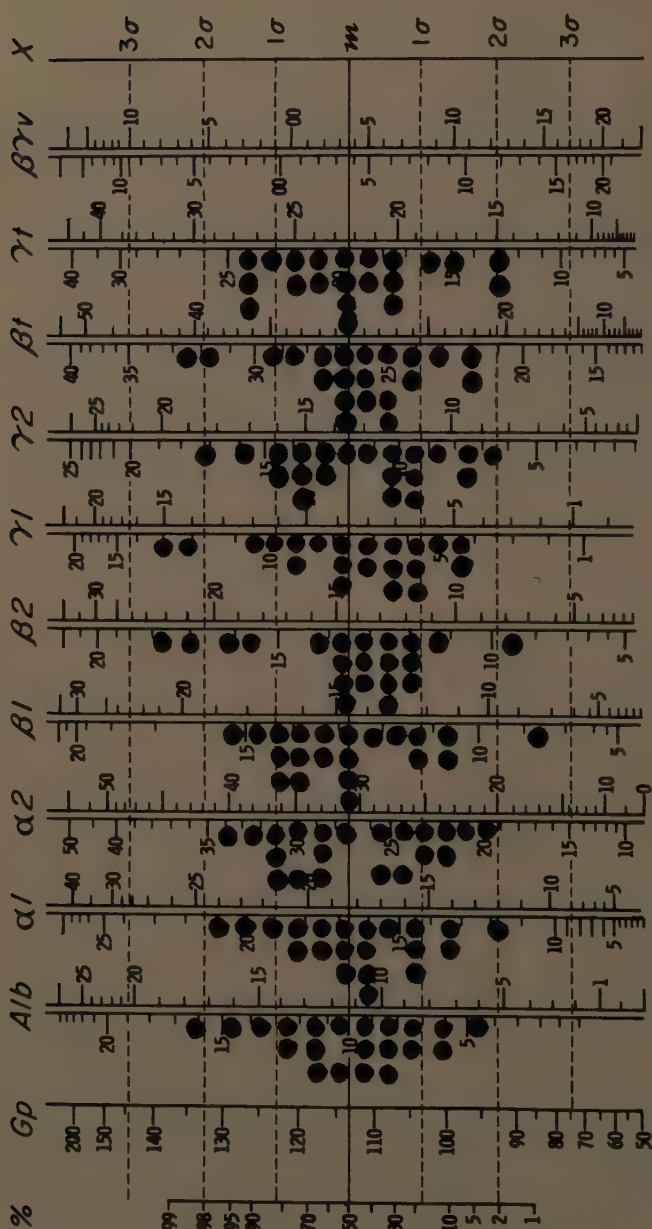


FIGURE 1. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) of 21 healthy adults.

electrophoretic fraction. The resultant electrophoretic profiles were then characterized according to their most prominent deviations from the normal mean (FIGURES 2 and 3).

Results

The most common electrophoretic abnormality was an increase in one or both of the alpha fractions, with a relative decrease in the albumin fraction,

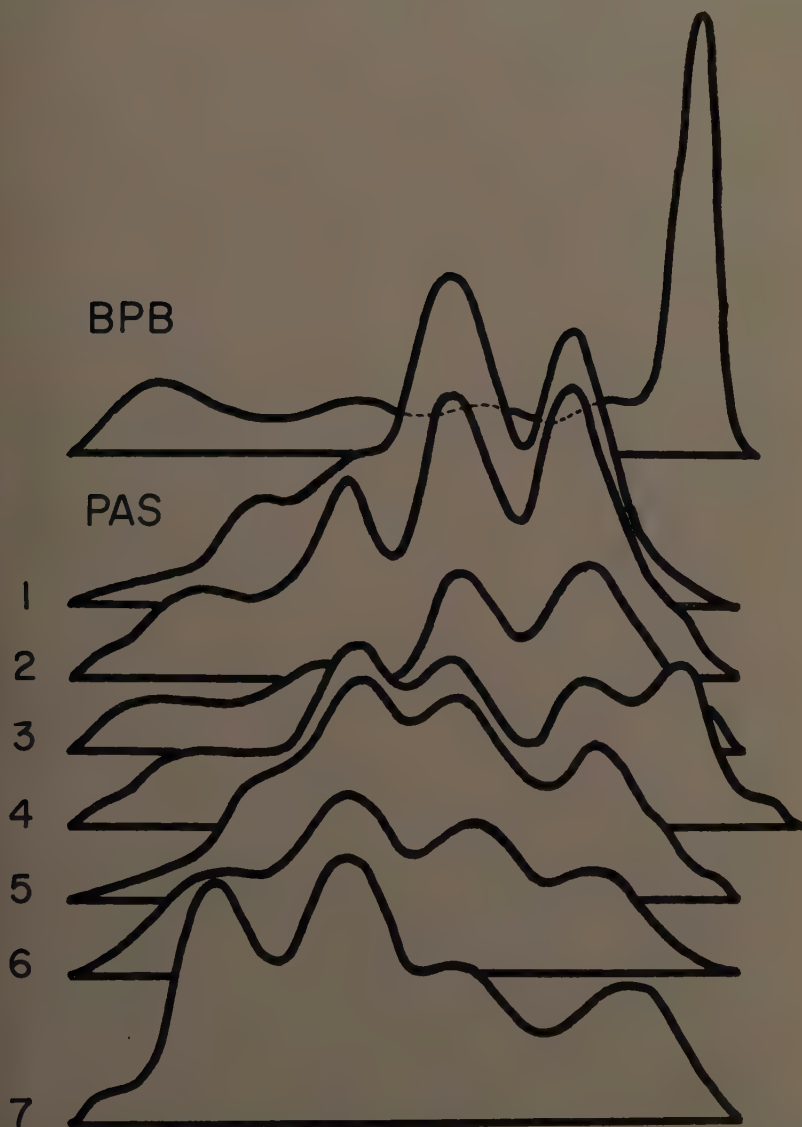


FIGURE 2. Tracings of abnormal serum glycoprotein electrophoretic patterns (PAS) compared to normal protein pattern (BPB).

and less marked changes in the other fractions (FIGURE 2, patterns 1, 2, and 3). This was observed in 138 of 303 patterns of patients with various diseases. The second most common pattern was characterized by an increase in the beta and gamma fractions, with only moderate changes in the other fractions (55

DIAGNOSIS, MEAN AND STANDARD DEVIATION	PULMONARY TUBERCULOSIS	COCCIDIOID- MYCOSIS	BRONCHOGENIC CARCINOMA	OTHER CARCINOMA	BLOOD-LYMPH- NEOPLASMS	MULTIPLE MYELOMA	DISCOID LUPUS ERYTHEMATOSUS	SYSTEMIC LUPUS ERYTHEMATOSUS	CIRRHOSIS	RHEUMATOID ARTHRITIS	PSORIATIC ARTHRITIS	PSORIASIS	ATOPIC DERMATITIS	ASTHMA	DIABETES	PHENYL- KETONURIA	MONGOLISM	TUBEROUS SCLEROSIS	n
HEALTHY ADULTS 113 ± 10	↑	↑	↑	↑	↑	↑	≡	↑	↑	↑	↑	↑	↑	≡	↑	↑	↑	↑	21
PULM. TUBERCULOSIS 132 ± 9.2		≡	↑	↑	≡	↑	≡	≡	≡	↑	≡	≡	≡	←	≡	≡	≡	≡	20
COCCIDIOIDOMYCOSIS 140 ± 31.3			↑	↑	≡	≡	←	≡	≡	≡	≡	≡	≡	←	≡	≡	≡	≡	26
BRONCHOGENIC CARCINOMA 177 ± 11.6				←	≡	≡	←	←	←	≡	←	←	←	←	≡	←	←	←	22
OTHER CARCINOMA 160 ± 29.0					←	≡	←	←	←	≡	←	←	←	←	≡	←	←	←	30
BLOOD-LYMPHATIC NEOPLASMS 153 ± 38.7						≡	←	≡	≡	≡	≡	≡	≡	←	≡	≡	≡	←	14
MULTIPLE MYELOMA 158 ± 34.9							←	≡	≡	≡	≡	←	≡	←	≡	←	≡	←	7
DISCOID LUPUS ERYTHEMATOSUS 115 ± 12.3								↑	≡	↑	↑	≡	≡	≡	↑	≡	≡	≡	7
SYSTEMIC LUPUS ERYTHEMATOSUS 136 ± 20.6									≡	↑	≡	≡	≡	←	≡	≡	≡	≡	14
CIRRHOSIS 136 ± 25.9										↑	≡	≡	≡	←	≡	≡	≡	≡	18
RHEUMATOID ARTHRITIS 159 ± 36.5											≡	←	≡	←	≡	←	≡	←	25
PSORIATIC ARTHRITIS 143 ± 27.7												←	≡	←	≡	≡	≡	≡	36
PSORIASIS 124 ± 17.7													≡	≡	↑	≡	≡	≡	17
ATOPIC DERMATITIS 129 ± 25.9														≡	≡	≡	≡	≡	7
ASTHMA 117 ± 14.9															↑	↑	↑	≡	20
DIABETES 144 ± 13.5																←	≡	≡	8
PHENYLKETONURIA 127 ± 15.1																	≡	≡	8
MONGOLISM 134 ± 29.9																		≡	9
TUBEROUS SCLEROSIS 127 ± 28.0																		15	n

FIGURE 3. Comparison of mean total serum glycoproteins of all diagnostic categories. The significance of the difference between the mean TSGp levels of any diagnostic categories is indicated by the symbol at the intersection of the diagnostic columns. In instances where the difference is significant at the 0.05 level, the arrow points in the direction of the diagnostic category having the higher mean TSGp level. Instances where the difference is not significant are indicated by the symbol ≡.

of 303 pathologic sera) as shown in FIGURE 2, pattern 7. The third most common pattern was characterized by a normal relative distribution, associated with an increase in total serum glycoprotein. This was observed in 28 of the 303 pathologic sera. Other abnormal patterns included those with the major relative increases in the beta and gamma-1 fractions (19 sera, FIGURE 2, pattern 6), in the alpha-2, beta, and gamma fractions (26 sera, FIGURE 2, pattern 5),

	TOTAL SERUM GLYCOPROTEIN		α -2 GLYCOPROTEIN (%)		INCIDENCE OF PATTERN TYPES									
	m	σ	m	σ	1,2,3	4	5	6	7	Nor- mal	Other	n		
PULMONARY TUBERCULOSIS	132	9.2	42.4	4.1	14	0	2	0	2	1	1	20		
COCCIDIOIDOMYCOSIS	140	31.3	44.4	18.1	18	3	1	0	1	2	1	26		
BRONCHOGENIC CARCINOMA	177	11.6	59.7	5.9	16	0	2	1	1	2	0	22		
OTHER CARCINOMA	160	29.0	52.3	12.7	18	3	3	0	3	0	3	30		
BLOOD-LYMPH. NEOPLASMS	153	38.7	40.7	4.7	7	1	1	0	2	1	2	14		
MULTIPLE MYELOMA	158	34.9	44.4	14.6	1	1	3	0	0	0	2	7		
DISCOID LUPUS ERYTHEMATOSUS	115	12.3	32.1	3.6	0	0	0	0	1	6	0	7		
SYSTEMIC LUPUS ERYTHEMATOSUS	136	20.6	36.9	9.7	3	1	0	0	8	2	0	14		
CIRRHOISIS	136	25.9	35.6	12.3	2	0	1	1	12	1	1	18		
RHEUMATOID ARTHRITIS	159	36.5	55.9	25.0	10	3	3	1	3	1	4	25		
PSORIATIC ARTHRITIS	143	27.7	44.7	12.6	18	1	3	3	2	7	2	36		
PSORIASIS	124	17.7	35.2	11.0	6	1	2	0	7	0	1	17		
ATOPIC DERMATITIS	129	25.9	35.8	12.2	2	0	0	1	4	0	0	7		
ASTHMA	117	14.9	37.8	8.7	7	0	0	9	2	0	2	20		
DIABETES	144	13.5	39.9	5.0	2	1	4	0	1	0	0	8		
PHENYLKETONURIA	127	15.1	37.5	10.1	4	1	0	2	1	0	0	8		
MONGOLISM	134	29.9	47.1	19.5	5	0	0	1	2	1	0	9		
TUBEROUS SCLEROSIS	127	28.0	32.4	12.8	5	1	1	0	3	4	1	15		
NORMAL ADULTS	113	10.0	27.0	3.9								21		

FIGURE 4. Mean total serum glycoprotein, mg, per cent α -2 glycoprotein, and incidence of electrophoretic pattern types for the diagnostic categories. The pattern types are shown in FIGURE 2.

BRONCHOGENIC CARCINOMA

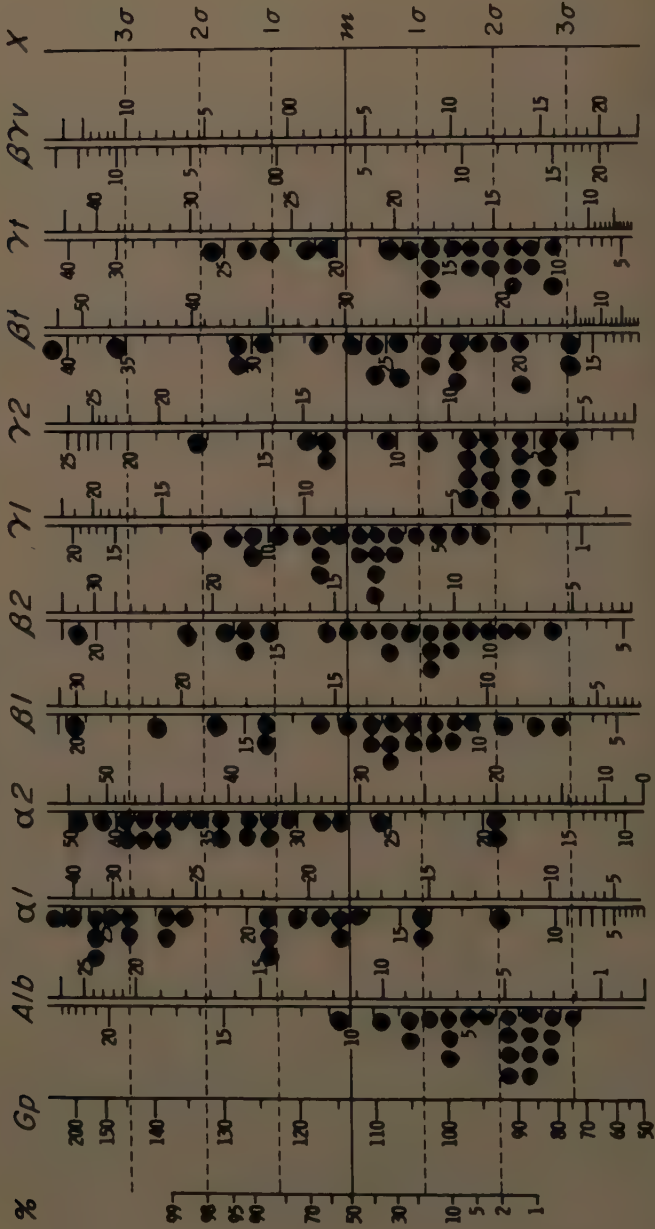


FIGURE 5. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in bronchogenic carcinoma.

and an increase in the albumin and/or prealbumin component associated with various changes in the other fractions (17 sera, FIGURE 2, pattern 4). Twenty patterns did not resemble any of the major types closely enough to warrant categorization.

The highest mean TSGp level was observed in bronchogenic carcinoma: 177 ± 11.6 mg. per cent (FIGURE 3). This was significantly higher than the mean levels of all other nonneoplastic diagnostic categories, with the exception of rheumatoid arthritis and diabetes. Most of this increase was in the alpha-1 and alpha-2 fractions (16 of 22 patients), although in 4 patients it was predominantly in the beta and gamma fractions (FIGURES 3 and 5).

The next highest mean TSGp level was observed in other types of carcinoma (160 ± 29 mg. per cent), which was significantly higher than all other categories, except bronchogenic carcinoma (177 ± 11.6 mg. per cent), rheumatoid arthritis (159 ± 36.5), multiple myeloma (158 ± 34.9 mg. per cent), and diabetes (144 ± 13.5 mg. per cent), none of which was significantly lower (FIGURE 3). With the exception of multiple myeloma, most of these patterns resembled those of the bronchogenic carcinoma group having the major increases in the alpha components (FIGURES 4, 5, 7, and 9).

The mean TSGp level in the group with coccidioidomycosis (140 ± 31.3 mg. per cent) was not significantly higher than observed in the pulmonary tuberculosis group (132 ± 9.2 mg. per cent). In addition, there was no significant incidence in the frequency of patterns with the major increase in the alpha fractions, indicating no significant differential diagnostic specificity to the glycoprotein changes in these two categories (FIGURES 10 and 11). There was, however, a substantial correlation between the degree of glycoprotein abnormality and the severity of the illness in the individual patients as judged by clinical and laboratory data.¹⁵

The most marked glycoprotein abnormalities, next to those observed in the neoplastic disease categories, were observed in rheumatoid arthritis, which had a mean TSGp level of 159 ± 36.5 mg. per cent (FIGURE 3). The most common abnormality was an absolute and relative increase in the alpha fractions: 10 of 25 patients (FIGURES 4 and 9), but the incidence of this pattern was less frequent than in bronchogenic carcinoma and coccidioidomycosis.

In rheumatoid and psoriatic arthritis the mean TSGp levels were 159 ± 36.5 and 143 ± 27.7 mg. per cent respectively, and there was no significant difference in incidence of patterns showing increases in the alpha fractions (FIGURES 4, 9, and 12). Although the mean TSGp level in psoriatic arthritis was higher than in psoriasis without arthritis (124 ± 17.7 mg. per cent), the latter level was significantly higher than normal.

Although the subjects with rheumatoid arthritis and systemic lupus occasionally shared certain clinical characteristics, the former had a mean TSGp significantly higher than the latter (136 ± 20.6 mg. per cent). In addition, the majority of patterns in systemic lupus erythematosus differed by having the greatest relative increase in the beta and gamma glycoprotein fractions (8 of 14 patients) as shown in FIGURES 4, 9, and 13. These abnormalities most closely resembled those observed in cirrhosis of the liver (FIGURE 14). The incidence of patterns showing the greatest relative increase in the beta and

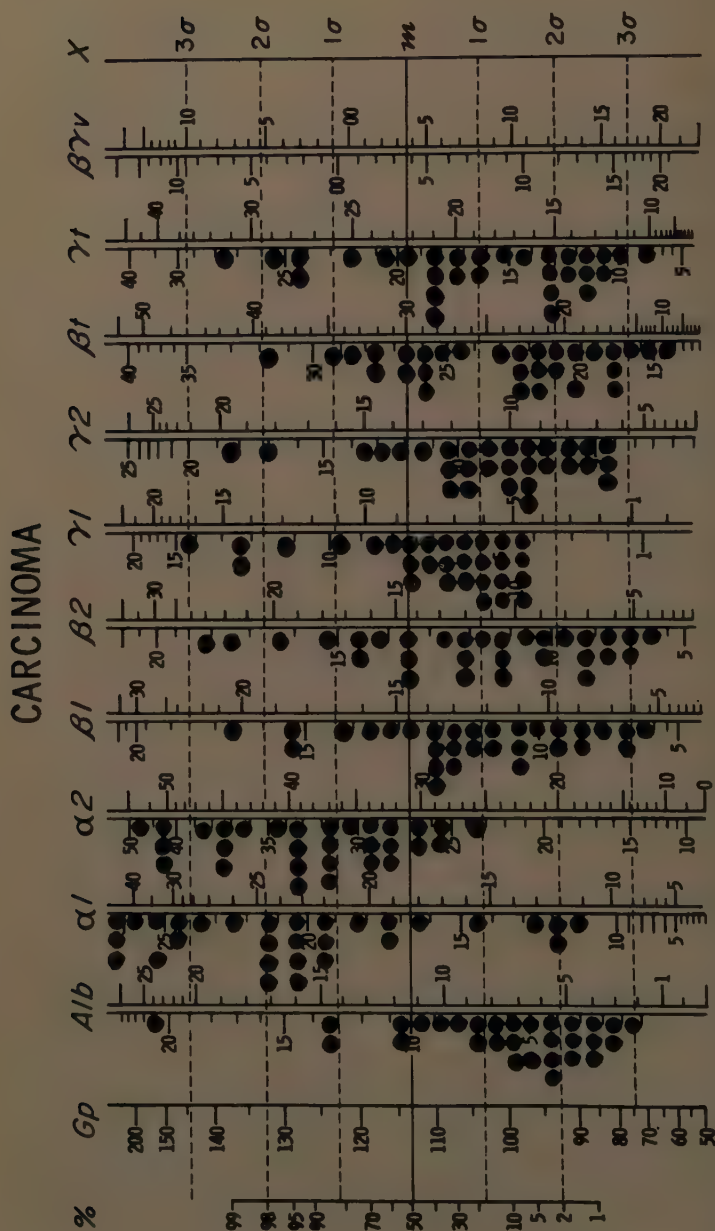


FIGURE 6. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in types of carcinoma other than bronchogenic.

MULTIPLE MYELOMA



FIGURE 7. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in multiple myeloma.

BLOOD-LYMPHATIC NEOPLASMS

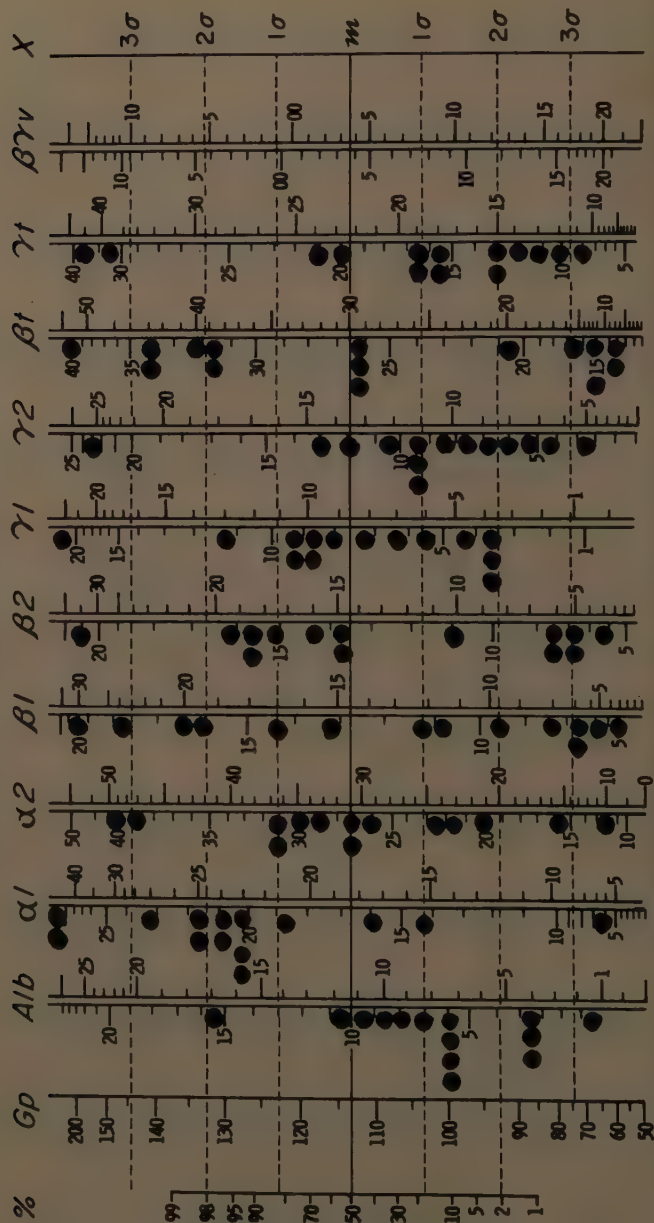


FIGURE 8. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in blood and lymphatic neoplasms.

RHEUMATOID ARTHRITIS



FIGURE 9. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in rheumatoid arthritis.

PULMONARY TUBERCULOSIS

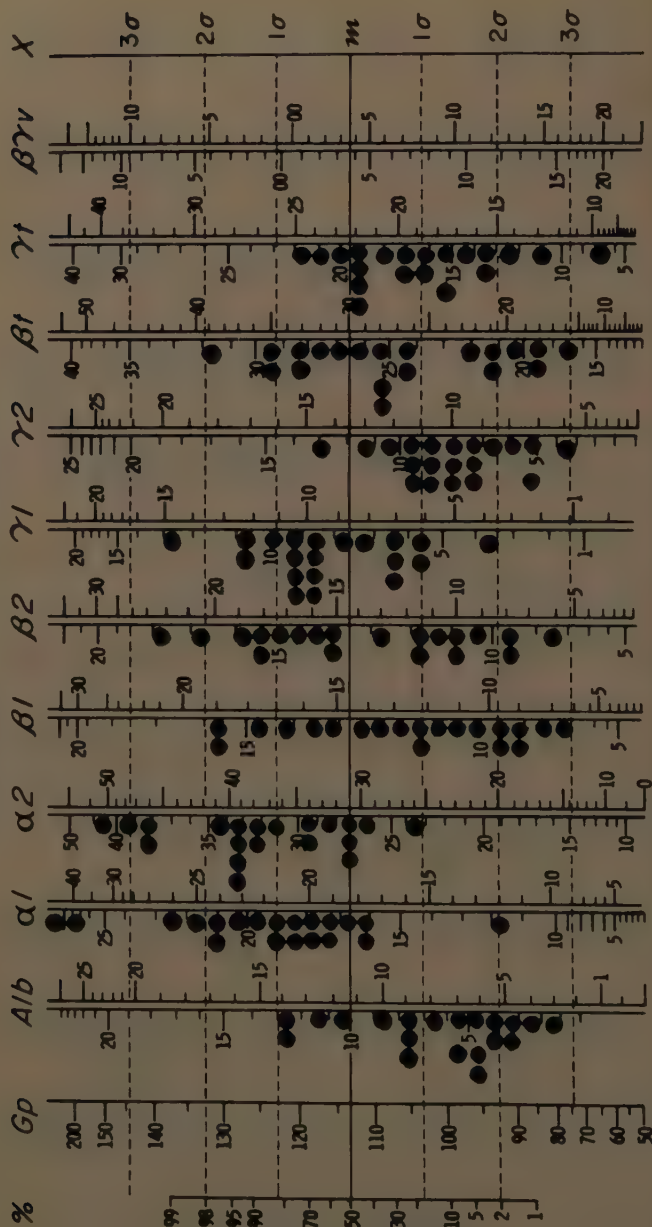


FIGURE 10. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in pulmonary tuberculosis.

COCCIDIOIDOMYCOSIS

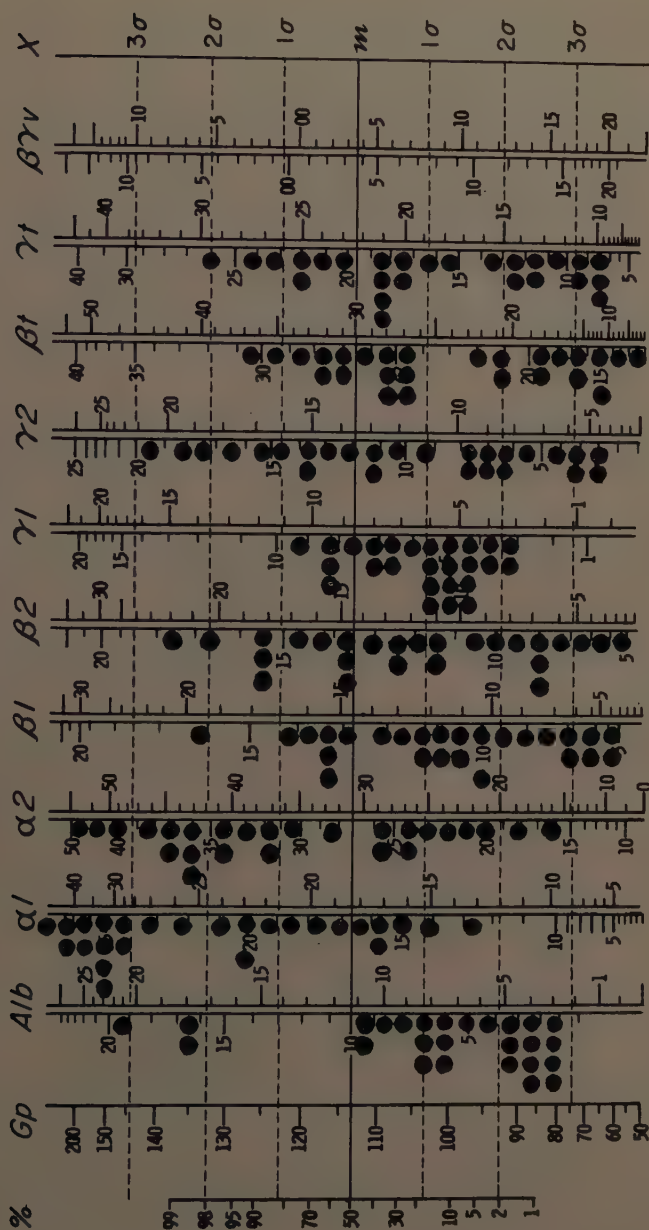


FIGURE 11. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in coccidioidomycosis.

PSORIATIC ARTHRITIS

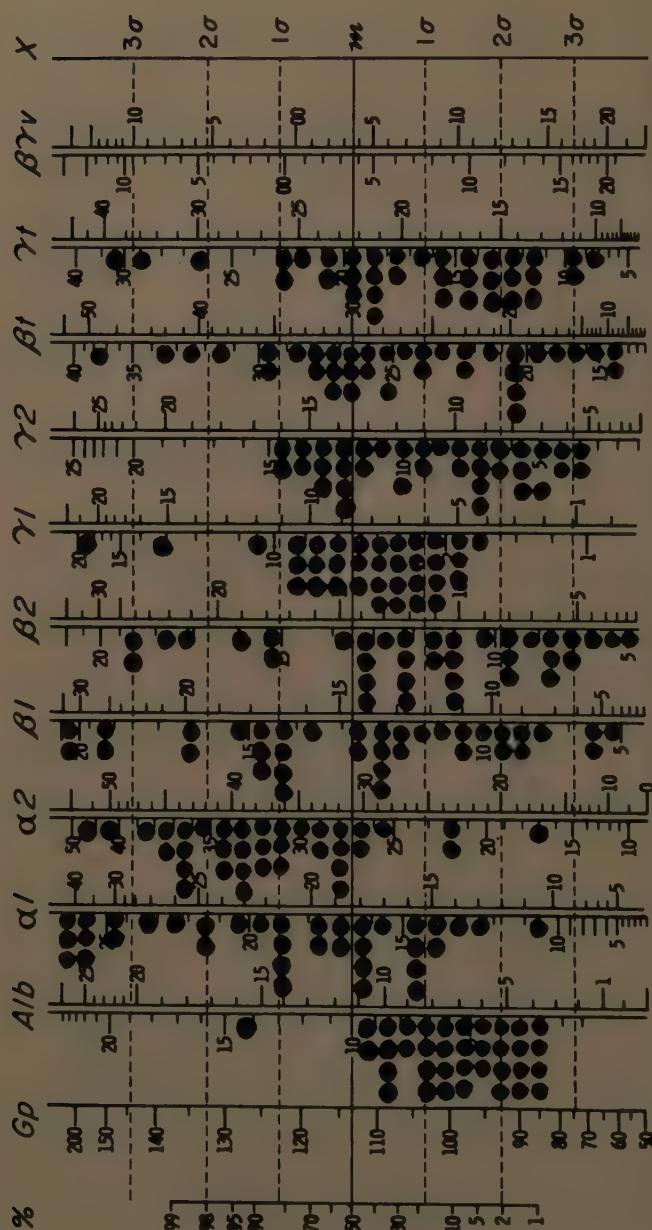


FIGURE 12. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in psoriatic arthritis.

SYSTEMIC LUPUS ERYTHEMATOSUS



FIGURE 13. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in systemic lupus erythematosus.

CIRRHOSIS

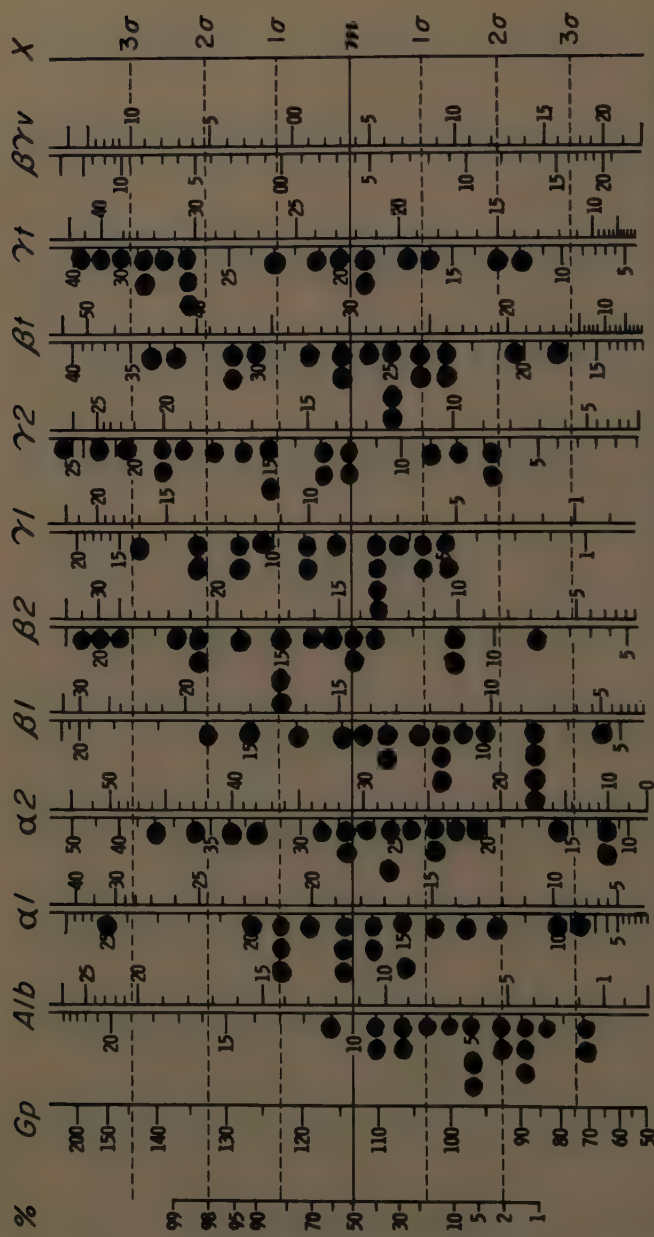


FIGURE 14. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in cirrhosis of the liver.

DISCOID LUPUS ERYTHEMATOSUS

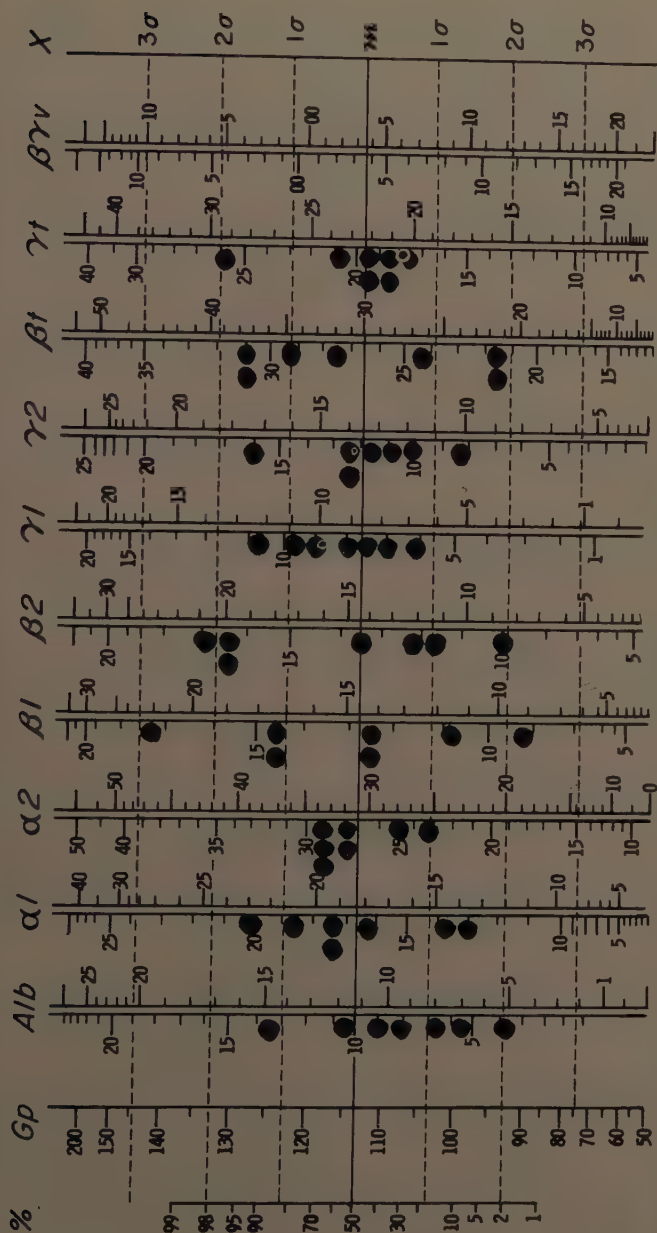


FIGURE 15. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in discoid lupus erythematosus.

BRONCHIAL ASTHMA

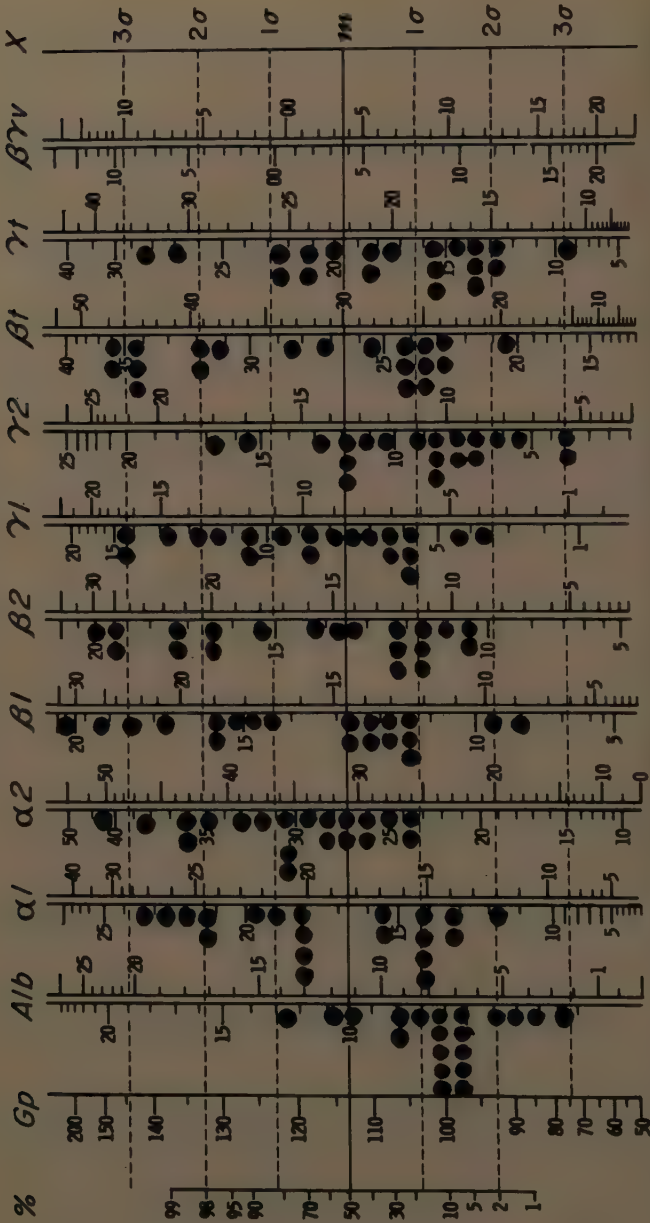


FIGURE 16. Group profile of glycoprotein electrophoretic fractions (relative per cent distribution) in bronchial asthma.

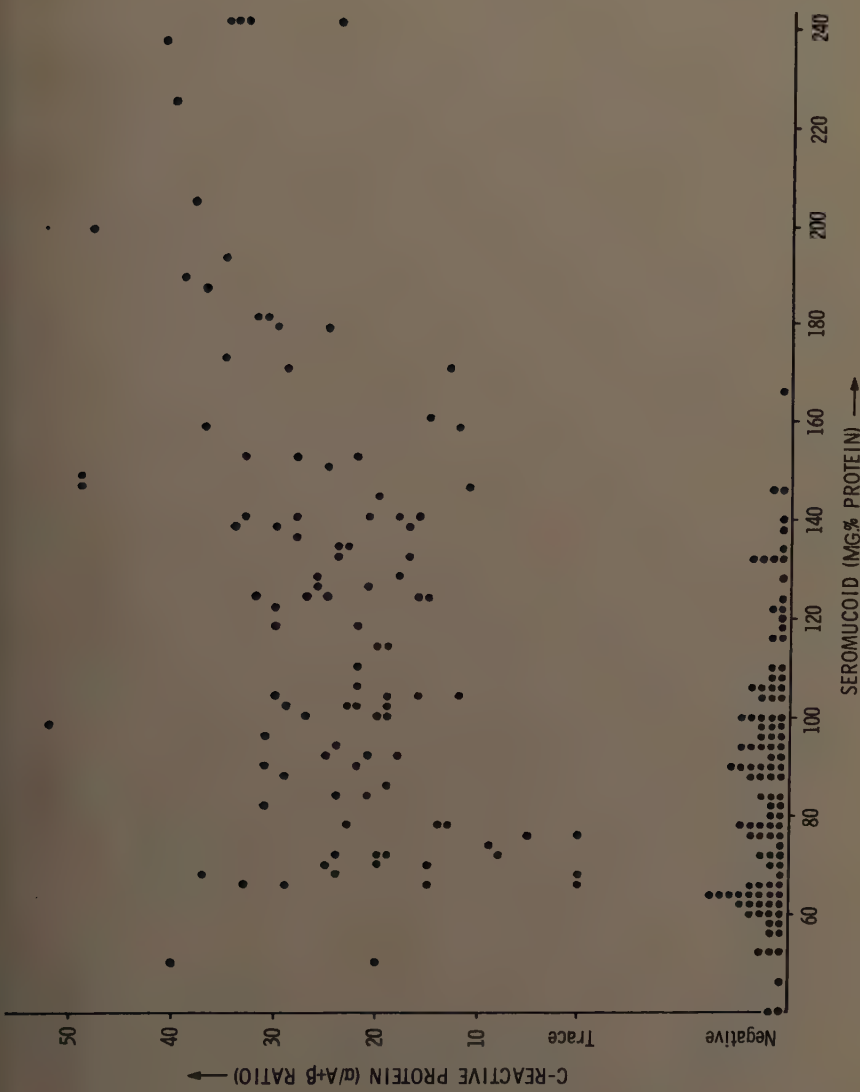


FIGURE 17. Correlation between seromucoid protein and C-reactive protein ($r = 0.42$).

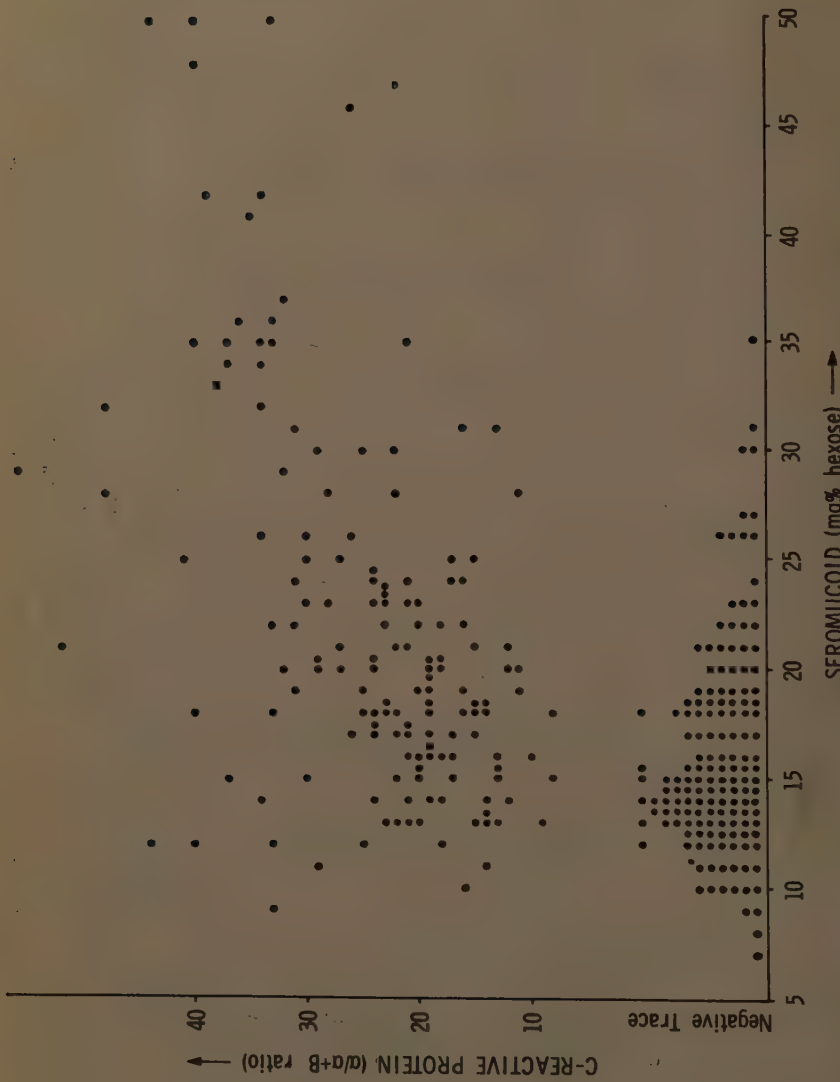


FIGURE 18. Correlation between seromucoid hexose and C-reactive protein ($r = 0.48$).

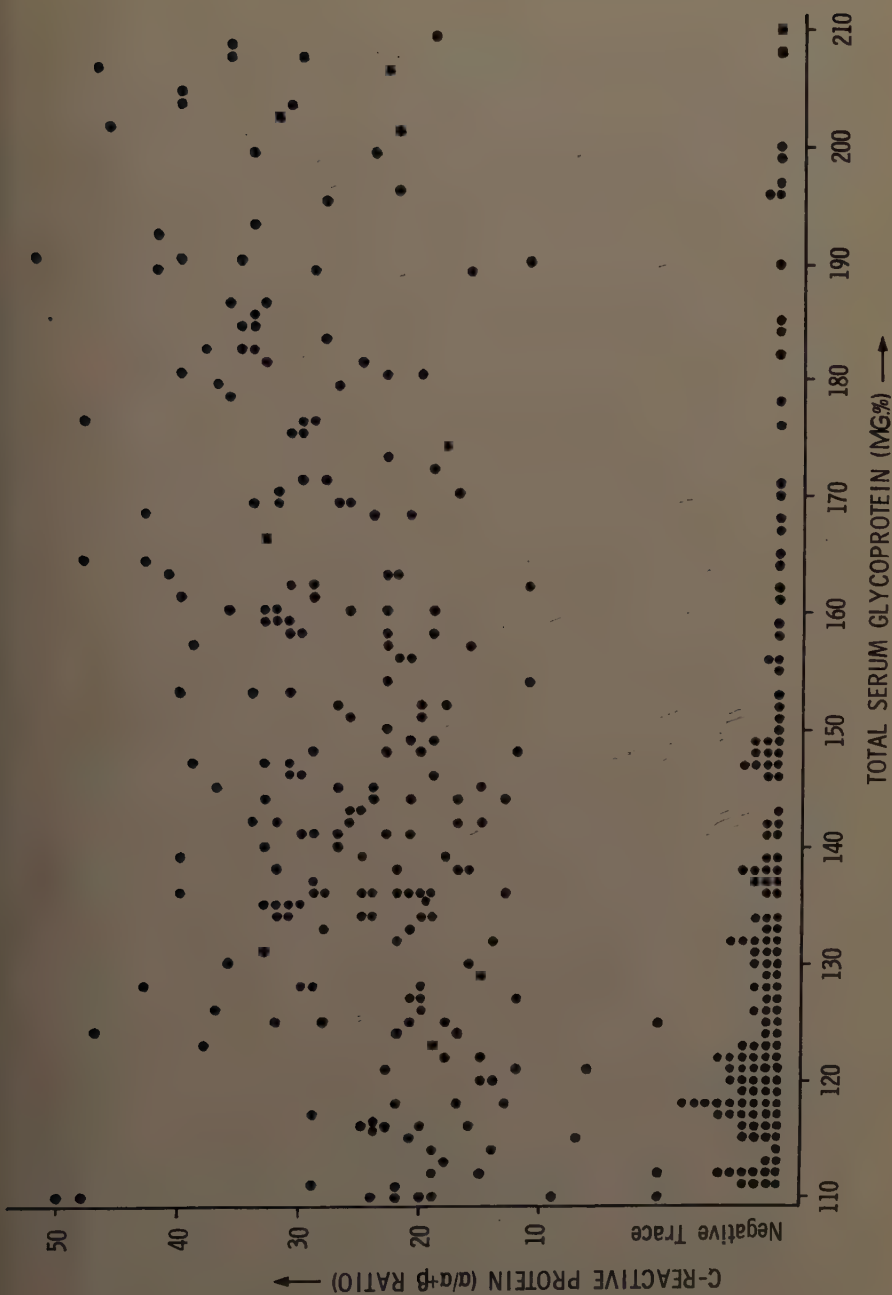


FIGURE 19. Correlation between total serum glycoprotein and C-reactive protein ($r = 0.42$).

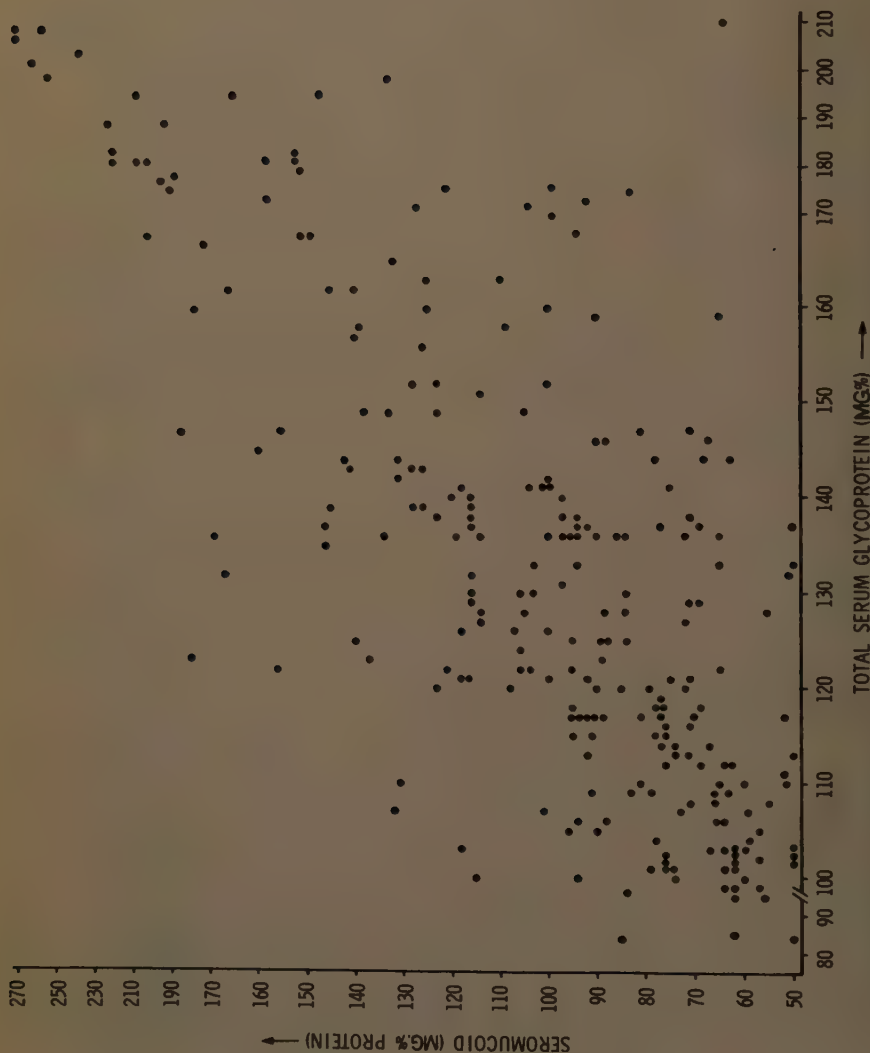


FIGURE 20. Correlation between total serum glycoprotein and seromucoid protein ($r = 0.75$).

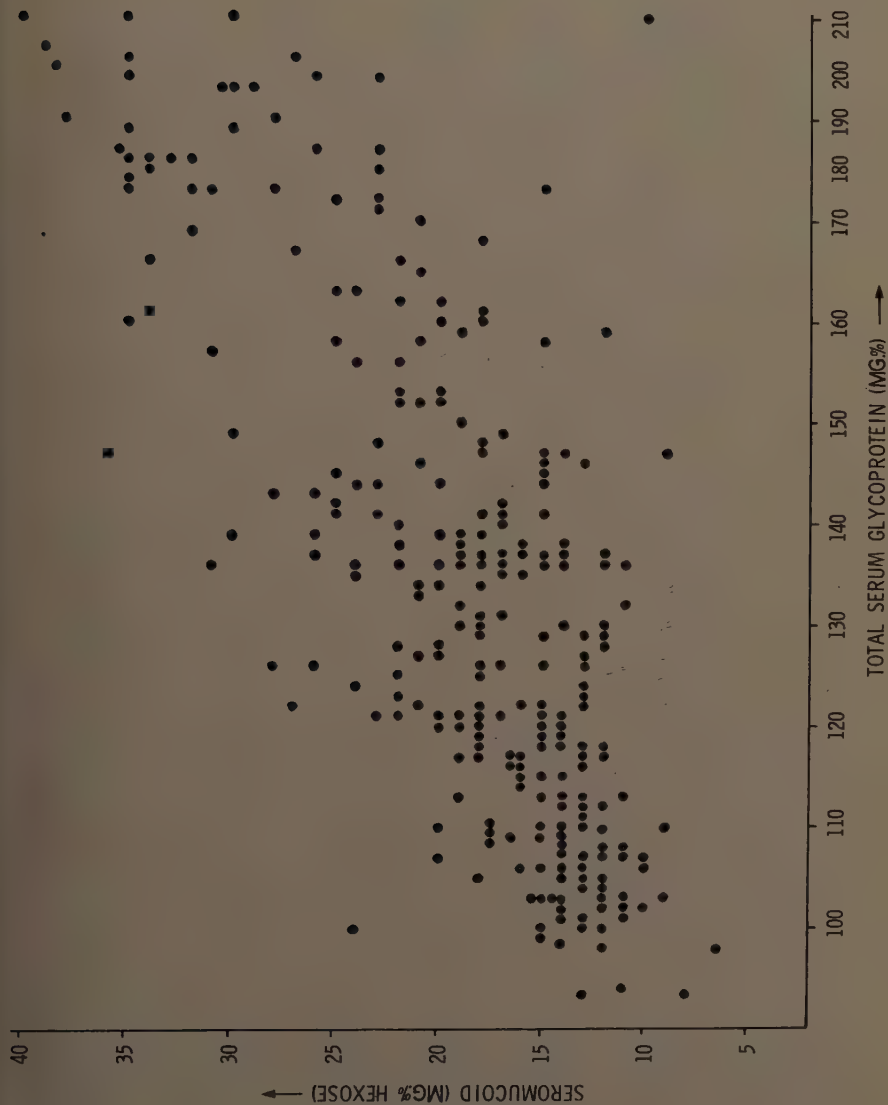


FIGURE 21. Correlation between total serum glycoprotein and serumucoid hexose ($r = 0.71$).

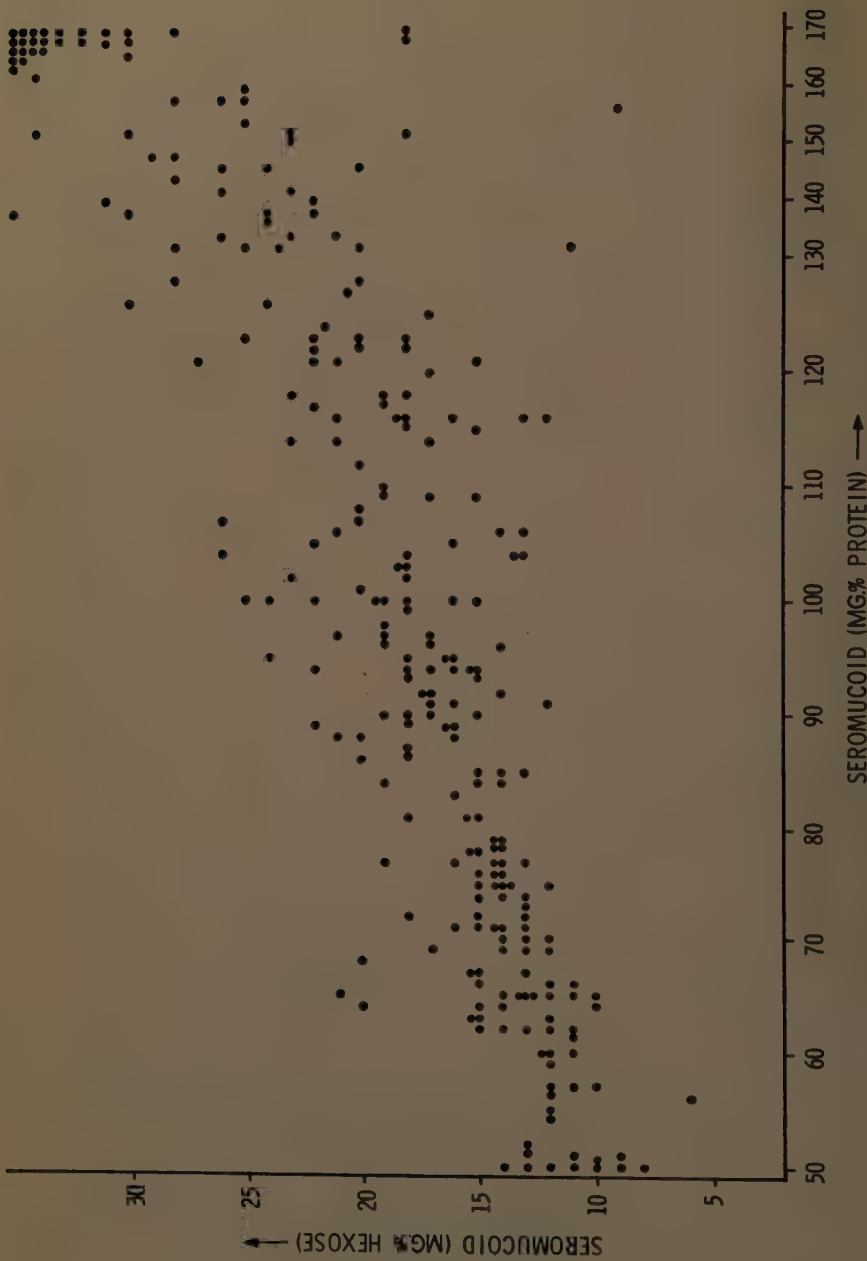


FIGURE 22. Correlation between seromucoid protein and seromucoid hexose ($r = 0.81$).

gamma fractions did not differ significantly in the systemic lupus erythematosus and cirrhosis categories (FIGURES 4, 13, and 14). The mean TSGp levels of these two categories were also similar (FIGURE 3).

As compared to the group with systemic lupus erythematosus, the group with discoid lupus erythematosus had a significantly lower MTSGp level (115 ± 12.3) which was not significantly higher than normal. The electrophoretic distribution was normal in 6 of 7 patients (FIGURES 4 and 15) in contrast to systemic lupus erythematosus, where only 2 of 14 patterns had a normal relative distribution of electrophoretic fractions.

The group with diabetes had a mean TSGp level of 144 ± 13.5 mg. per cent, which was significantly higher than that observed in the healthy adult, discoid lupus erythematosus, psoriasis, asthma, and phenylketonuria. The mean level was surprisingly high, however, in view of the fact that these patients were selected because of their freedom from complications such as poor control, infection, clinically evident vascular disease, or acidosis.

The remaining groups with psoriasis, atopic dermatitis, phenylketonuria, mongolism, and tuberous sclerosis showed few significant quantitative differences with respect to their mean TSGp levels or qualitative differences with respect to their electrophoretic patterns.

In all of these categories except asthma, the electrophoretic abnormalities were usually limited to moderate increases in the alpha fractions and a decrease in the albumin fraction. In asthma, however, an unusually frequent abnormality was an increase in the beta and gamma-1 fractions (FIGURES 4 and 16). This was observed in 9 of 20 patients, whereas it was observed in only 10 of 303 patterns in the other diagnostic categories.

The levels of the glycoprotein, seromuroid protein, and seromuroid hexose were compared to the levels of C-reactive protein, an acute phase reactant widely used clinically as a measure of the inflammatory response.

It was observed that when C-reactive protein was present, the levels correlated most closely with the seromuroid hexose ($r = 0.48$) as in FIGURE 18. There was less correlation between C-reactive protein and seromuroid protein ($r = 0.42$) as shown in FIGURE 17, and total serum glycoprotein ($r = 0.42$) as in FIGURE 19. Seromuroid protein showed a closer correlation with total serum glycoprotein ($r = 0.75$), as in FIGURE 20, than did seromuroid hexose ($r = 0.71$) as in FIGURE 21. Seromuroid protein and seromuroid hexose showed the closest correlation ($r = 0.81$) as may be seen in FIGURE 22.

Discussion

The data of this study indicate that the quantitative and qualitative changes in serum glycoproteins for discrete diagnostic categories of neoplastic, infectious, inflammatory, metabolic, and congenital disease show many significant differences. Although these differences are not usually distinct enough in the individual patient to be utilized as isolated diagnostic data, they indicate that the glycoproteins do not respond in an entirely nonspecific and uniform manner to diverse pathologic stimuli.

The most common abnormality was an absolute and relative increase in one or both of the alpha fractions associated with a decrease in the albumin fraction. This showed a good correlation with the severity of the clinical signs of

inflammation in the individual patients. Striking exceptions to this generalization, however, were observed in individual patients with bronchogenic and other carcinomas, diabetes, and tuberous sclerosis, where there were little or no clinically evident inflammatory complications to account for these changes.

Because of superficial clinical similarities it was expected that the glycoprotein abnormalities in rheumatoid arthritis and systemic lupus erythematosus would be similar. There were marked quantitative and qualitative differences between the two, however, indicating a potential diagnostic application in the differential diagnosis of an atypical arthritis. The pattern types and quantitative levels closely resembled those observed in cirrhosis of the liver.

The group with bronchial asthma had a mean TSGp level that was not significantly higher than that of the healthy adult group. This was not unexpected, inasmuch as the patients in this group were clinically free of infectious complications and relatively asymptomatic at the time of testing. One unexpected finding, however, was the frequent small but significant increases in the beta and gamma-1 electrophoretic fractions. This type of abnormality was not frequent or distinct enough to be useful for diagnostic purposes, but its infrequency in the other diagnostic categories suggests that further study of this fraction in asthma may be of value.

Paper electrophoresis is a relatively crude method for studies of the individual glycoproteins of serum. This is particularly true for the alpha fractions, where the observed value may be the resultant of the individual variations in several immunologically discrete components. It is likely that diagnostic specificity will be greatly improved when it is possible to perform quantitative measurements of these individual proteins using immunologic techniques now under development.

Summary

The quantitative and electrophoretic changes in serum glycoproteins in discrete diagnostic categories of neoplastic, infectious, inflammatory, metabolic, and congenital diseases show significant differences indicating that glycoproteins do not respond in a uniform and nonspecific manner to diverse pathologic stimuli.

The highest mean TSGp levels were observed in neoplastic disease and rheumatoid arthritis and, although not diagnostic, were significantly higher than the levels in most other diagnostic categories.

The most frequent electrophoretic abnormality was an increase in the alpha fractions, although in systemic lupus erythematosus and cirrhosis the most frequent abnormality was an increase in the beta and gamma fraction, and in bronchial asthma an increase in the beta and gamma-1 fractions.

The TSGp showed a closer correlation with seromuroid protein ($r = 0.75$) than with seromuroid hexose ($r = 0.71$), and the C-reactive protein level showed a closer correlation with seromuroid hexose ($r = 0.48$) than with TSGp ($r = 0.42$).

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SERUM PROTEIN FRACTIONS AND ANTIBODY STUDIES IN GNOTOBIOTIC* ANIMALS REARED GERMFREE OR MONOCONTAMINATED†

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The germfree animal has been described as differing in various aspects from animals reared under conventional animal room conditions.¹ These differences have for the most part been ascribed to the respective absence or presence of an associated microbial flora. The role played by specific members of the normal flora in establishing the "physiologic state" of conventional life is obscured by the complex nature of the normal flora. This paper describes some observations made on the serum protein fractions and antibacterial agglutinins found in animals maintained under less complex conditions; that is, either entirely germfree or in association with only single selected strains of intestinal microorganisms (monocontaminated). In another paper,‡ the response of the germfree rat to a purified antigen, bovine serum albumin, is described.

Earlier work on the comparison of serum proteins of germfree and conventional rats has characterized the germfree rat as having lower but nevertheless detectable levels of α_2 , β , and γ -globulin fractions.^{2,3} A similar comparison in chickens showed the principal difference to be a decreased γ -globulin level under germfree conditions.³ The characterization of other germfree-reared species from this standpoint, for example, mouse, guinea pig, rabbit, sheep, and goat have been reported and reviewed by Wostmann.^{3,4} In general, all germfree-reared animal species studied to date have shown varying degrees of hypogammaglobulinemia, depending on the species, age and, possibly, dietary factors.

Germfree rat and chicken serum have been found to be devoid of many, but not necessarily all, antibacterial agglutinins demonstrable in the serum of conventional animals.^{4,5} The agglutinins that have been demonstrated in germfree animals are believed to be formed in response to dead bacteria and probably other antigenic substances indigenous to the autoclaved diet. These dietary antigens may thus be responsible, at least in part, for the levels of the probable antibody-carrying serum protein fractions seen in germfree animals.

Exposure of young adult germfree rats to the normal intestinal flora of conventional rats resulted in a gradual increase in various globulin fractions.⁶ The increase occurred first in the α_2 fraction at one week post-contamination followed by an increase in β globulin by the second week. γ -globulin stayed at the low germfree level and only started to rise after a two to

* A gnotobiotic animal may be defined as an animal reared free of association with other organisms, that is, germfree, or reared in the presence of only specifically known associates.

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‡ "Recent Studies on the Plasma Proteins of Germfree Animals," by B. S. Wostmann, elsewhere in these pages.

four week period. Agglutinins for *Escherichia coli* were not demonstrated in germfree rats or in rats one week post-contamination. However, they were detected after two weeks when the beta globulins were on the increase but prior to any appreciable rise in gamma globulin. Gustafsson *et al.*⁷ have also reported a four week lag in the rise of gamma globulins in growing germfree rats after exposure to a normal flora. Gamma globulins eventually reached conventional levels after seven weeks post-contamination.

The previous work has illustrated that the germfree animal has at least some degree of immunologic competence through exposure to nonviable dietary antigenic stimuli while still in the germfree state. Furthermore, exposure of previously germfree animals to contamination with a complex conventional flora results in a correction of the deficiency of serum protein fractions believed to carry antibodies. The observations reported here were made on monocontaminated animals in order to study the response of the host to contamination with selected microorganisms under specified bacteriological conditions. Part of the monocontaminated experiments were run in conjunction with studies on dental caries in the rat. The choice of *Lactobacillus casei* (a strain isolated from human dental caries) as one of the monocontaminants was made on this basis. *Streptococcus faecalis* (a rat strain) and *Clostridium perfringens* (types A and E) were the other monocontaminants used. Lactobacilli and enterococci are considered to be representative genera most prevalent in the intestinal flora of conventional animals. From this quantitative standpoint, they were considered to be potential factors responsible for differences seen between germfree and conventional life. However, *L. casei* is not one of the *Lactobacillus* species normally found in the intestinal tract of conventional Lobund rats. This consideration is discussed later in relation to agglutinins found in conventional rats. *C. perfringens* was selected on the basis that conventional chickens fed penicillin displayed certain "germfree-like" characteristics and concomitantly showed a virtual elimination of clostridia from the intestinal flora to be the most prominent microbiological change.^{10,12}

Methods

Germfree rats and chickens were reared in steel isolator units according to the methods of Reyniers *et al.*^{5,8} Monocontamination of the animals was carried out by passing a pure culture of the desired microorganism into the isolator via a germicidal trap and swabbing the mouth of each germfree animal with a cotton applicator dipped into the culture. Monocontamination was established at birth or after three to five weeks of germfree life, depending on the particular experiment. The animals were held in the gnotobiotic state for the desired length of time; their microbiologic status was checked periodically and terminally.⁹ The electrophoretic analysis of the serum was by the Antweiler microelectrophoresis technique as slightly modified by Wostmann *et al.*⁶ Agglutinin titers were determined as described by Reyniers *et al.*⁵

Results and Discussion

Groups of germfree rats were inoculated per os at weaning with a pure culture of bacteria and maintained in the monocontaminated state. The effects of the various monocontaminants on the serum protein fraction levels and agglutinin

titers are shown in TABLE 1. Only the α_2 -, β -, and γ -globulin levels are presented here since the foregoing has already indicated that these were the fractions most likely to change if antibodies were to be produced in response to monocontamination. Germfree and conventional levels are also presented for comparison. The germfree data confirm earlier observations^{2,3} that the germ-free rat shows a deficit in α_2 , β , and γ globulins when compared to

TABLE 1
SERUM GLOBULIN FRACTIONS AND ANTIBACTERIAL AGGLUTININS IN RATS REARED
GERMFREE, CONVENTIONALLY, OR MONOCONTAMINATED* WITH *CLOSTRIDIUM*
PERFRINGENS, *STREPTOCOCCUS FAECALIS* OR *LACTOBACILLUS CASEI*†
Number of Animals in Parentheses

Category	Serum globulin fractions mg./100 ml.			Agglutinin titers‡	
	Alphas	Beta	Gamma	Monocontam- inated	Conventional
Germfree	58 ± 5	299 ± 14	126 ± 9 (16)	—	—
Conventional	188 ± 25	591 ± 34	523 ± 52 (12)	—	—
Monocontaminated					
<i>C. perfringens</i> , type A (9 weeks environment only)	65 ± 3	346 ± 21	150 ± 17 (5)	16-32 (5)	0 (4)
<i>C. perfringens</i> , type E (9 weeks environment only)	61 ± 4	309 ± 21	134 ± 21 (6)	16-32 (6)	0 (4)
<i>S. faecalis</i> (21 weeks en- vironment only)	86 ± 6	353 ± 26	147 ± 13 (5)	32-256 (6)	2-256 (44)
<i>S. faecalis</i> † (21 weeks environment plus 2 × parenteral injection)	85	239	152 (2)	256-512 (3)	—
<i>L. casei</i> (22 weeks en- vironment only)	52 ± 13	287 ± 25	139 ± 14 (3)	32-128 (3)	0 (13)
<i>L. casei</i> † (22 weeks en- vironment plus 3 × parenteral injection)	86 ± 4	332 ± 29	176 ± 20 (4)	64-128 (4)	—

* All rats were reared germfree for 3 to 5 weeks prior to monocontamination per os. Time maintained in the monocontaminated environment indicated in parentheses along with parenteral treatment where applicable.

† Some of the animals in the monocontaminated groups were also challenged parenterally with the homologous organism. Parenterally treated animals were given weekly intraperitoneal injections of homologous organism superimposed on the environmental monocontaminant. Animals were bled 1 week after the last injection.

‡ Reciprocal of titer range for organism homologous to monocontaminated category as tested in conventional and monocontaminated rats. Germfree rat titers were uniformly negative.

the conventional rat. Germfree rat serum did not contain agglutinins for any of the organisms used as monocontaminants in the other groups.

In all monocontaminated experiments, the organisms became well established in the intestinal tract, reaching populations of log 9 to log 10 bacteria per gram of dry cecal contents. Monocontamination with *Clostridium perfringens* (types A or E), *Streptococcus faecalis* or *Lactobacillus casei* resulted in the appearance of fair to good serum agglutinin titers against whole-cell suspensions. However, attempts to demonstrate precipitins in the *S. faecalis* rat serum against the

Lancefield Group D polysaccharide component extracted from *S. faecalis* cells were negative. The most striking observation was that the appearance of agglutinins in the rats after monocontamination was not accompanied by any appreciable rise in globulins. These remained at the low germfree levels. The rat's response to monocontamination with the organisms used was sufficient to produce an antibody of good enough quality to be detected by standard agglutination procedures. However, there was apparently insufficient quantity to bring about an appreciable change in the agglutinin carrying fraction(s) as measured by the relatively crude quantitation of the electrophoretic analysis.

TABLE 2*

ELECTROPHORETIC PATTERNS AND ANTIBACTERIAL AGGLUTININS IN THE SERUM; SCATTERED RETICULOENDOTHELIAL CELLS IN THE WALL OF THE LOWER ILEUM OF GERMFREE, CONVENTIONAL, AND MONOCONTAMINATED CHICKENS AGED 65 TO 70 DAYS
UNLESS OTHERWISE INDICATED
Number of Animals in Parentheses

Category	Serum protein fractions mg./100 ml.				Agglutinin titers†		Total scattered‡ Rt. cells in ileum wall per sq. mm.
	Albumin	Alpha ₁	Alpha ₂ & Beta	Gamma	Monocon- taminated	Conven- tional	
Germfree	3041 ± 101	95 ± 11	362 ± 20	319 (7) ± 29	—	—	930 (11) ± 60
Conventional	2833 ± 48	122 ± 9	490 ± 27	701 (12) ± 48	—	—	2860 (11) ± 240
Monocontaminated§ <i>Streptococcus</i> <i>faecalis</i>	2489 ± 117	89 ± 11	414 ± 49	770 (8) ± 113	32-128 (5)	8-32 (9)	1460 (6) ± 150
<i>Clostridium per-</i> <i>fringens</i> (type A)	2830 ± 106	86 ± 12	475 ± 31	534 (10) ± 33	0-4 (10)	0-2 (13)	2000 (3) ± 215

* Portions of this table were previously published in *Antibiotics Annual* 1959-1960 (10).

† Reciprocal of titer range shown for monocontaminated and conventional animals against organism homologous to the monocontaminated group. Germfree chicken titers were all uniformly negative.

‡ See Gordon and Bruckner-Kardoss¹¹ for technical details. Results taken from approximately equal numbers of 35- and 65-day-old chickens.

§ All chicks monocontaminated at time of hatching and maintained throughout life in monocontaminated state.

Attempts were made to increase the agglutinin titer and agglutinin-carrying globulin fractions by injecting parenteral antigen over the amount available through natural routes in the monocontaminated groups. Parenteral injection of homologous organism (5×10^7 to 5×10^9 per dose) in the *S. faecalis* or *L. casei* rat groups did not cause any appreciable rise in titers or in globulin levels over that observed in monocontaminated but uninjected animals. It appeared that agglutinin production was being maintained at near maximum level by antigens supplied from the external milieu alone.

Germfree chickens were monocontaminated at hatching with the same strains of *S. faecalis* or *C. perfringens* type A used in the rat. The results on serum protein fractions and agglutinin titers are presented in TABLE 2. The data show that monocontamination with *S. faecalis* resulted in the appearance of homologous agglutinins. However, in the case of the chicken, the appearance

of agglutinins was accompanied by a rise in gamma globulins to levels comparable to conventional birds. However, a comparison between the amount of additional globulin produced and the observed agglutinin titer would indicate that only a small part of the additional gamma globulin was capable of agglutinating a suspension of *S. faecalis*. It is probable that some of the globulin response was directed against subsurface antigens or metabolic products of the bacteria that would not be detected by the agglutination technique employed. On the other hand, polysaccharide extracts from *S. faecalis* (Lancefield Group D), readily precipitated by commercially prepared rabbit antiserum, did not show the presence of precipitins (by ring test) in the monocontaminated-chicken serum.

Monocontamination with *C. perfringens* in the chicken resulted in only a moderate rise in gamma globulin to a level intermediate between that seen in germfree versus conventional chicks. Little or none of the additional globulin was detected as agglutinating antibody for *C. perfringens* (6 of 10 chicks showed no agglutinin titers at the low 1:2 serum dilution). None of the monocontaminated chicken sera displayed any appreciable antilecithinase activity for the alpha toxin of *C. perfringens*.

In recapitulation at this point, *C. perfringens*, *L. casei*, or *S. faecalis* as monocontaminants in the rat can stimulate a fair-to-good antibody response when measured in terms of agglutinin titer. However, the appearance of agglutinin was not reflected by any appreciable increase in serum globulins as measured by electrophoretic techniques. Gustafsson *et al.*⁷ have also reported that rats monocontaminated with *Staphylococcus albus* maintained low gamma-globulin levels over a 10-week period. The lack in globulin response is somewhat surprising in view of the fact that exposure of young adult previously germfree rats to the full complement of microbiota of conventional rats results in a total correction of the hypoglobulinemic state.^{6,7} This is presumed to be due to the formation of antibody in response to the complex of microbial antigens. Yet *L. casei*, *C. perfringens*, or *S. faecalis* which, as monocontaminants, individually establish intestinal populations equal to the total count of all bacteria cultivated from the intestinal contents of conventional rats, nevertheless fail to raise the globulin level. This was obviously not true for the chicken. All monocontaminants studied to date have caused a rise in gamma globulins above germfree levels. With *S. faecalis* or *Staphylococcus aureus*, the levels have equaled or exceeded that seen in conventional chicks. *C. perfringens* caused only a moderate rise in gamma globulin intermediate between germfree and conventional values.

The response in the chicken to monocontamination with *C. perfringens* or *S. faecalis* is further exemplified by changes produced in the wall of the lower ileum (these data were previously published¹⁰ and are incorporated as part of TABLE 2). The total scattered reticuloendothelial (RE) cell count in the ileum wall of conventional chicks was much higher than in germfree. The difference was mostly in terms of the lymphocytes and plasma cells in the subepithelial tissue and of Schollen leukocytes in the epithelium. Other RE elements (monocytes, macrophages, heterophils, basophils, and eosinophils) were not appreciably affected. Monocontamination with *C. perfringens* brought about a marked increase in those RE cells deficient under germfree conditions, while *S. faecalis* at best stimulated only a mild response.

The work with monocontaminated chickens has thus indicated certain differences in response to different organisms. *S. faecalis* seemed to be more active in stimulating the humoral responses of the host while giving only a "sub-conventional" although obvious increase in the number of scattered RE cells of the intestinal wall. With *C. perfringens*, on the other hand, the emphasis was more on the cellular than on the humoral response. These results point up the differences not only between the two host species, rats and chickens, in response to the same microorganisms but also point up the differences produced by different organisms in the same host.

A brief comment is needed regarding the agglutinin titers in conventional rats against organisms used in the monocontaminated experiments. In general, conventional animal serum is able to agglutinate a variety of microorganisms found in the intestinal tract.^{4,5} In TABLE 2, conventional rats showed considerable titers for *S. faecalis*, one of the more prevalent members of the normal intestinal flora. No titers were observed for *C. perfringens* or *L. casei*. However, *C. perfringens* constitutes only a small fraction of the total microbial population in the conventional rat, 10^2 to 10^3 per gram dry cecal contents. Under monocontaminated conditions, this organism reached a population of 10^9 per gram and stimulated perceptible agglutinin formation. The difference between absence or presence of anti-*C. perfringens* agglutinins in these two categories might be due to the difference in quantity of specific antigenic stimulus available. A similar explanation may hold for *L. casei*. While conventional rats harbor large numbers of lactobacilli in the intestinal flora, *L. casei* was not among the lactobacillus species found in the normal flora of the conventional animal colony. This may account for the absence of these specific agglutinins. *L. casei*, however, is antigenic under monocontaminated conditions where large numbers of intestinal *L. casei* are maintained.

During the course of this work, some of the *L. casei* and *S. faecalis* monocontaminated rats were allowed to breed into the second generation. The first and second generations differed in that the former represented animals with a history of previous germfree generations. They were inoculated per os at weaning after having been suckled germfree for three weeks and thus had no previous exposure to viable organisms during their prenatal and suckling period. The second-generation rats were the progeny of the above and thus were exposed to the monocontaminants at birth by contact with the mother and environment. Presumably there could have been prenatal influences as well. Results on serum protein fractions and agglutinin titers are shown in TABLE 3. First generation data are repeated from TABLE 1 for comparative purposes.

Particularly interesting was the finding that second-generation rats monocontaminated with *L. casei* (group C) failed to develop agglutinins for the homologous organism while first-generation rats (group A) did. This confirmed an earlier report⁴ on a group of 25 second- and third-generation *L. casei* monocontaminated rats that also failed to form agglutinins for this organism. The phenomenon was referred to as similar to an acquired immunologic tolerance for *L. casei*. However, in the earlier work, the animals were not stimulated parenterally with the homologous organism as a test for immunologic tolerance. In the present experiments, first- and second-generation rats were each split into 2 littermate groups. Groups B and D received 3 intraperitoneal injections

of viable *L. casei* (approximately 5×10^7 to 5×10^9 cells per dose) superimposed over the normal exposure to the lactobacilli in the environment. Comparison of groups C and D indicate that second-generation rats had only a simulated immunologic tolerance since the parenteral injection of *L. casei* in group D resulted in antibody formation. It thus appears that little or no tolerance existed to the lactobacillus antigens at those antibody producing sites accessible through parenteral treatment. The animals in group C acted more as though the antigen could not reach potential antibody producing sites via natural routes if contact with the organism was first made at or possibly before birth. The mechanism by which this "simulated tolerance" occurs is at present obscure.

TABLE 3

THE LACK OF AGGLUTINATING ANTIBODY RESPONSE IN SECOND GENERATION *L. CASEI* MONOCONTAMINATED RATS AS COMPARED TO RATS MONOCONTAMINATED WITH *S. FAECALIS*: SOME ANIMALS IN EACH GROUP BEING CHALLENGED PARENTERALLY* WITH THE HOMOLOGOUS ORGANISM
Number of Animals in Parentheses

Group	Category†	Serum globulin fractions mg./100 ml.			Agglutinin titer‡
		Alpha ₂	Beta	Gamma	
Mono.— <i>L. casei</i>					
A	1st gen. (environ. only)	52 ± 13	287 ± 25	139 ± 14 (3)	32-128 (3)
B	1st gen. (environ. + 3 inject.)	86 ± 4	332 ± 29	176 ± 20 (4)	64-128 (4)
C	2nd gen. (environ. only)	69 ± 21	318 ± 15	99 ± 4 (3)	0 (3)
D	2nd gen. (environ. + 3 inject.)	75 ± 16	374 ± 36	293 ± 14 (3)	128-1024 (4)
Mono.— <i>S. faecalis</i>					
E	1st gen. (environ. only)	86 ± 6	353 ± 26	147 ± 13 (5)	32-256 (6)
F	1st gen. (environ. + 2 inject.)	85	239	157 (2)	256-512 (3)
G	2nd gen. (environ. only)	88 ± 1	276 ± 16	166 ± 32 (3)	256-2048 (3)
H	2nd gen. (environ. + 2 inject.)	159 ± 27	377 ± 41	241 ± 54 (4)	512-4096 (4)

* Animals given indicated number of weekly intraperitoneal injections of homologous organisms superimposed over monocontaminating organism in environment. Animals were bled 1 week after last injection.

† See TABLE 1 for germfree and conventional rat data.

‡ Reciprocal of titer range.

Another interesting finding was the observation that parenteral injection of *L. casei* superimposed on the homologous environmental monocontaminant had no appreciable effect on an already functioning antibody-forming system in first-generation groups (A and B). As stated earlier, agglutinin formation seemed to be progressing at a near-maximum level by exposure to external milieu antigen alone. In second generation animals, the parenteral injection of *L. casei* in group D animals activated a previously quiescent system to produce not only detectable agglutinin but also a higher shift in gamma globulin above germfree levels.

The phenomenon of simulated immunologic tolerance as seen with *L. casei* could not be repeated with *S. faecalis* as monocontaminant (groups E, F, G, H). An interesting question is raised regarding the role lactobacilli may play in

nature in their association with the host since lactobacilli are generally the first organisms to become established in the intestinal tract of the newborn. It may be that lactobacilli play an important role in immunologic phenomena during the early stages in life. In this connection, Gillespie *et al.*¹³ have reported the apparent lack of agglutinins for lactobacilli in human sera despite the fact that these organisms make up the major part of the human intestinal flora. This could be a similar phenomenon in humans.

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Part IV. Genetic, Induced, Nutritional, and Environmental Factors Affecting the Plasma Proteins

INHERITED VARIATIONS IN HUMAN SERUM PROTEINS: STUDIES ON THE GROUP-SPECIFIC COMPONENT*

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During the last few years inherited variations of human serum proteins have been shown by several different methods. Starch gel electrophoresis has enabled the separation of genetically determined variations in haptoglobins^{1,2} and transferrins.^{3,4} Serological techniques have disclosed inherited variations among the gamma globulins.⁵ This study however is concerned with another serum protein with inherited variations, an α_2 globulin not related to the haptoglobins, which was first described by Hirschfeld in 1959,⁶ and called the group-specific component (Gc).⁷

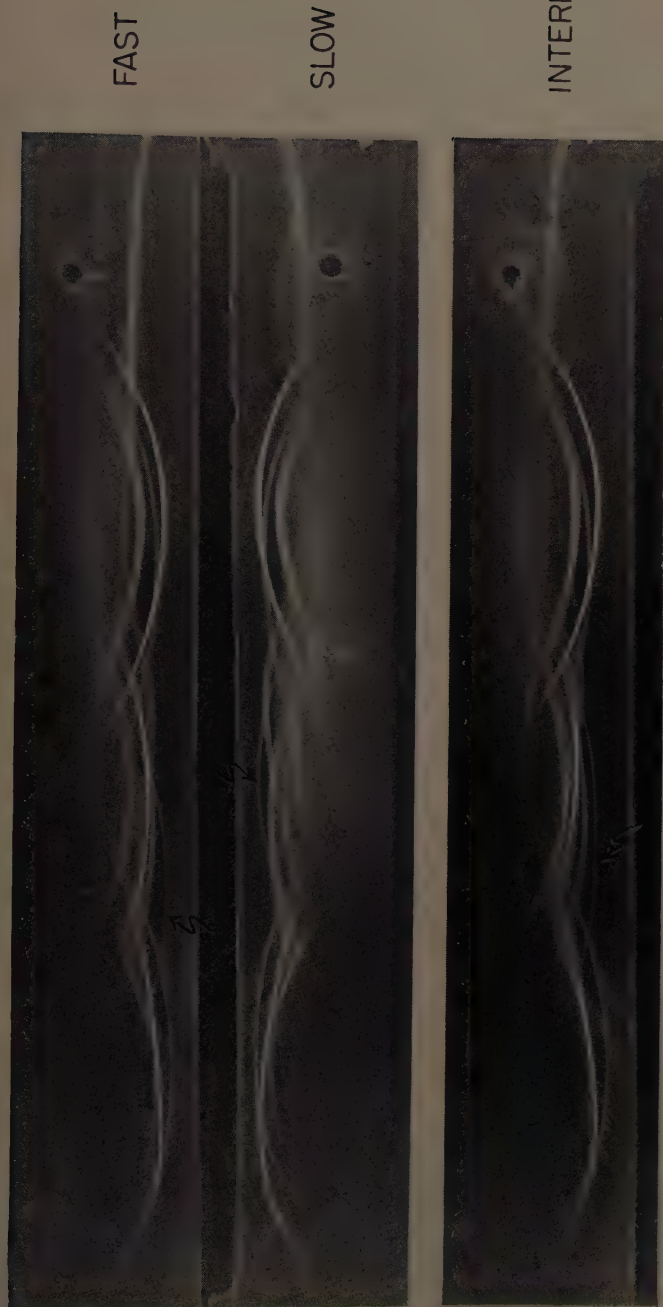
Thus far the group-specific component has been demonstrated only by the technique of immunoelectrophoresis described by Grabar and Williams.⁸ In the present study a modification⁹ of Scheidegger's microtechnique¹⁰ using small glass slides was utilized.

The immunoelectrophoretic analysis of three different normal human sera is illustrated in FIGURE 1. An antinormal human serum produced in the horse was used that yielded 18 precipitation lines. In the α_2 -globulin region, differences in the position of one precipitation line were observed. In some sera a fast-moving component was observed (FIGURE 1, top). In others the precipitate took the position of a slow-moving component (FIGURE 1, center). A third somewhat flattened precipitation line was observed in some sera that did not completely overlap the two other precipitation lines (FIGURE 1, bottom). Under ideal conditions, this precipitation line showed a double peak where the position of the two peaks corresponded to the fast and slow-moving components. It was possible to show by absorption and mixing experiments that these proteins were immunologically indistinguishable. FIGURE 2 illustrates the three different phenotypes of the group-specific component and the positions of the precipitates of albumin, α_2 macroglobulin, and transferrin. The three different phenotypes may be called the fast, slow, and intermediate (two peaked) type. All normal human sera examined thus far may be classified into one of these three groups.

Evidence has been presented by Hirschfeld *et al.*¹¹ that the observed variations in the mobility of the group-specific component are under genetic control. The genetic hypothesis has been further investigated by studies on families and twin pairs. The results of a study of 31 families with 78 offspring confirmed the inheritance as a two allelic system without dominance (TABLE 1). The fast-moving type represents one homozygote with the genotype 1-1 and the slow-moving type represents the other homozygote of the genotype 2-2. The intermediate type represents the heterozygote with the genotype 2-1.

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Group - specific α_2 -Component
Different phenotypes in normal human serum



Anti human horse serum 13412 (Institut Pasteur)

FIGURE 1. Immuno-electrophoretic analysis of three different normal human sera illustrating the three phenotypes. The precipitation arcs of the group-specific component are indicated by arrows.

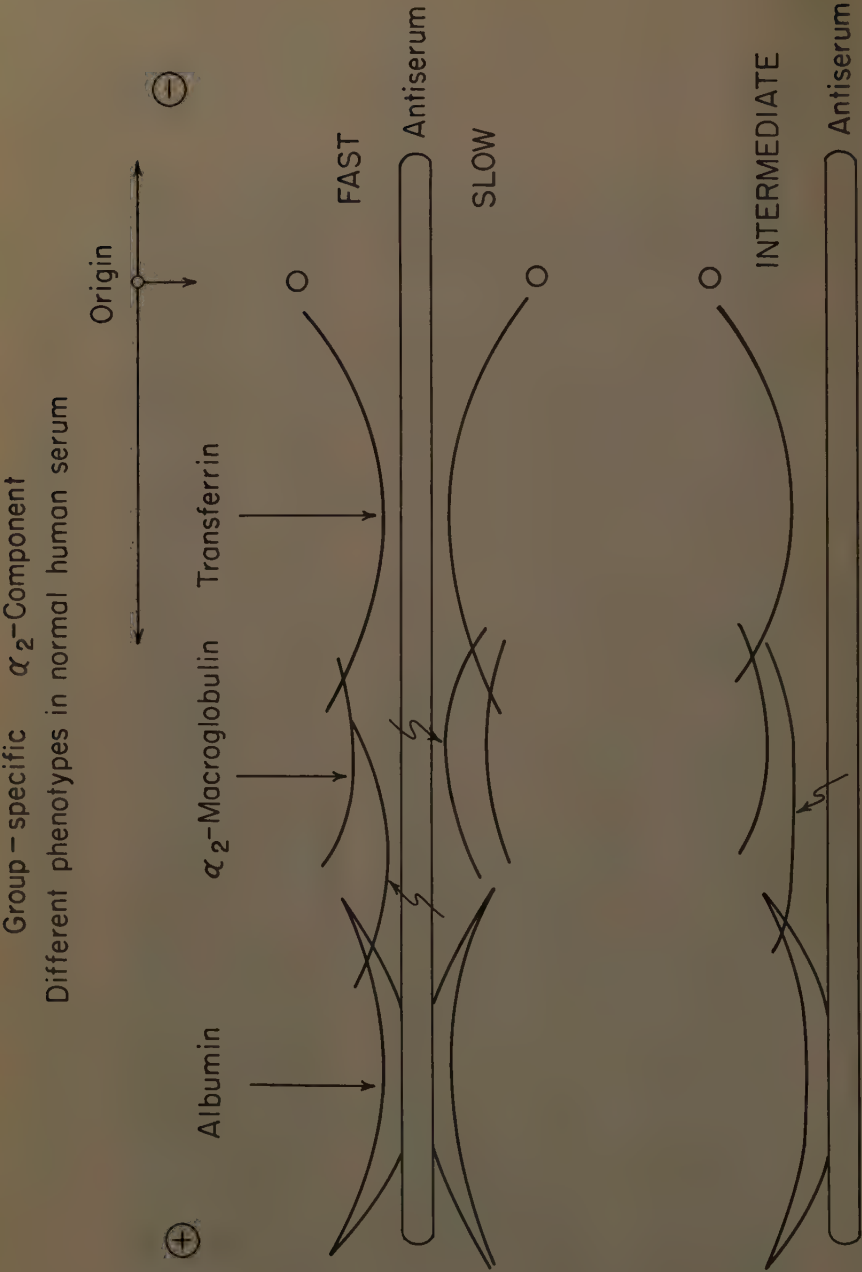


FIGURE 2. Schematic illustration of the three phenotypes in relation to the positions of other serum proteins.

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The group-specific component was examined in 34 twin pairs. In 17 monozygotic twin pairs the Gc types were concordant, but in 17 dizygotic twins only 13 were concordant (TABLE 2).

The genotype frequencies in a population of 86 unrelated white individuals of the United States (New York, N.Y.) are given in TABLE 3. The frequency

TABLE 1
DISTRIBUTION OF THE GROUP-SPECIFIC α_2 COMPONENT IN THIRTY-ONE FAMILIES

Parental phenotypes		No. of families	Offspring			Total
			Fast	Inter.	Slow	
Fast	Fast	11	32	—	—	32
Fast	Inter.	13	19	14	—	33
Fast	Slow	3	—	5	—	5
Inter.	Inter.	3	3	3	1	7
Inter.	Slow	1	—	1	—	1
Slow	Slow	0	—	—	—	—
		31	54	23	1	78

TABLE 2
GROUP-SPECIFIC α_2 COMPONENT IN THIRTY-FOUR TWIN PAIRS

	Concordant	Discordant	Total
Monozygotic	17	0	17
Dizygotic	13	4	17

TABLE 3
GENOTYPE FREQUENCIES (HARDY-WEINBERG)

U. S. Whites	Fast 1-1		Inter. 2-1		Slow 2-2		Total	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Observed	48	55.82	32	37.21	6	6.97	86	100
Expected	47.6	55.35	32.8	38.10	5.6	6.55	86	100

$$\chi^2 = 0.044; P > .8.$$

TABLE 4
GENE FREQUENCIES IN SEVERAL POPULATIONS

Population	Fast 1-1		Inter. 2-1		Slow 2-2		Total	p	q	s'
	No.	Per cent	No.	Per cent	No.	Per cent				
U. S. whites	48	55.82	32	37.21	6	6.97	86	0.744	0.256	0.033
U. S. Negroes	98	81.67	19	15.83	3	2.50	120	0.896	0.104	0.019
Navajo Indians	188	95.43	8	4.06	1	0.51	197	0.975	0.025	0.008

Key: p = gene frequency of Gc¹ (fast gene); q = gene frequency of Gtc² (slow gene); and s' = standard error of the gene frequencies.

Group-specific α_2 component
Relative mobilities of the different phenotypes
in starch block electrophoresis

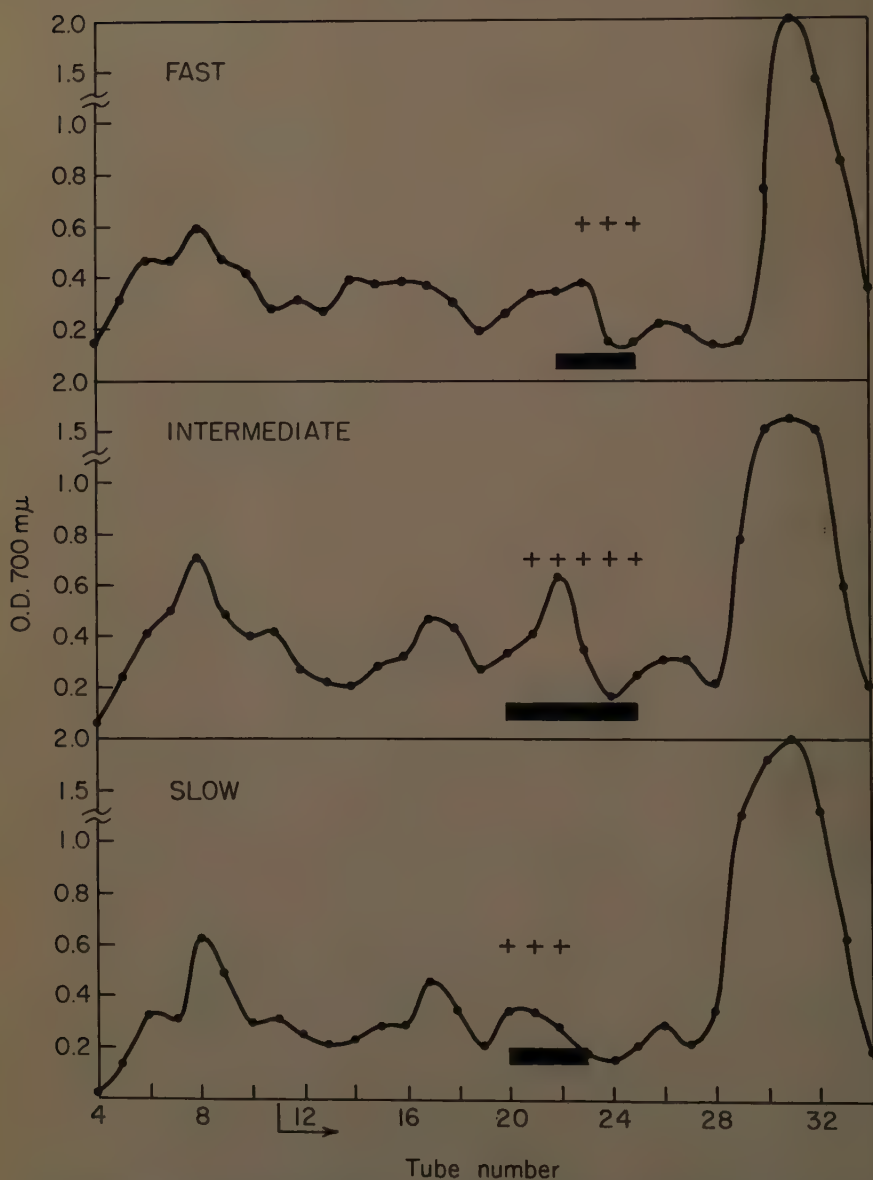


FIGURE 3. A simultaneous electrophoretic separation on a starch block of serum from the three phenotypes. The crosses indicate the presence of the group-specific component tested by immunoelectrophoresis of the fractions. The solid bar indicates the presence of the group-specific component tested by absorption (details described in text).

of the fast gene (Gc^1) was found to be 0.744 and of the slow gene (Gc^2) 0.256. The χ^2 test for goodness of fit of the observed distribution with the expected distribution according to the Hardy-Weinberg equilibrium demonstrated good agreement. These results were not statistically different from those obtained by Hirschfeld *et al.* in a Swedish population.¹¹

The distribution of the genes was determined in two different populations (TABLE 4). In 120 unrelated United States Negroes (New York, N.Y.) the slow gene (Gc^2) was more rare than in the United States white population, but not as rare as in 197 Navajo Indians from Arizona. The standard errors of the estimates for the gene frequencies were small. The χ^2 test of homogeneity was performed, and indicated that the observed gene frequencies in the 3 populations were significantly different from each other.

When equal amounts of serum from the 2 homozygotes 1-1 and 2-2 were mixed *in vitro*, immunoelectrophoretic analysis revealed a precipitation line of the intermediate type that was indistinguishable from that seen in normal

TABLE 5
SOLUBILITY OF THE GROUP-SPECIFIC α_2 COMPONENT

Method	Precipitate	Supernatant
$(NH_4)_2SO_4$ 33.3%	—	+
50%	+	—
Rivanol 0.4% 1:1	—	+
2:1	+	—
PO_4 0.02 M pH 5.4	—	+
0.005 M pH 5.8	—	+
Perchloric acid 0.6 M		—
Trichloroacetic acid 5 %		—

heterozygotes (2-1). The precipitation line of the group-specific component in the mixture of the two homozygotes showed the reaction of identity. Immunological identity was also observed in the heterozygote. After absorption of an antiserum with the α_2 fraction of serum, obtained by preparative starch block electrophoresis, the Gc precipitation line disappeared. The disappearance of the precipitation line occurred when the antiserum was absorbed with an α_2 fraction from a slow type and tested with a fast type serum, and also when an antiserum absorbed with a fast type α_2 fraction was tested against a serum of slow type. Thus far, using immunoelectrophoresis and nonspecific antisera, it has not been possible to detect immunological differences between the three phenotypes.

The precise nature of the group-specific component is unknown and only preliminary chemical studies have been performed. The relative electrophoretic mobilities of the different types of the Gc were studied in preparative starch block electrophoresis (FIGURE 3). The component was determined either by immunoelectrophoretic analysis of the eluted and concentrated starch block fractions or by testing antisera absorbed with the various fractions against normal human sera. The latter technique, although time consuming,

is more sensitive. The relative mobilities of the group-specific component in starch block electrophoresis were similar to those observed in agar.

The solubility characteristics of this protein were obtained by studying whole serum (TABLE 5). The group-specific component is not a euglobulin. It is neither soluble in 0.6 *M* perchloric acid nor in 5 per cent trichloroacetic acid. It was precipitated by 50 per cent ammonium sulphate but remained in the supernatant at 33.3 per cent saturation. Rivanol (6.9 diamino-2 ethoxyacridine) in a 0.4 per cent solution also precipitated the protein when 2 ml. of Rivanol was added to 1 ml. of serum. When the Rivanol serum mixture was in a 1:1 ratio the protein remained in the supernatant.

Summary

(1) The inheritance of the group-specific α_2 component has been established as a two allelic system without dominance, confirming the results of Hirschfeld.

(2) Significant differences in gene frequencies have been observed in a United States white population, in a United States Negro population, and in Navajo Indians.

(3) Studies of the solubility characteristics of the group specific-component have been performed.

Acknowledgments

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THE EFFECTS OF PROTEIN DEPLETION AND REPLETION ON THE CONCENTRATION AND DISTRIBUTION OF SERUM PROTEINS AND PROTEIN-BOUND CARBOHYDRATES OF THE ADULT RAT*

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The first unequivocal evidence that diet affects the regeneration of serum proteins was reported by Kerr *et al.* in 1918.^{1,2} The observations of these investigators have stimulated numerous studies concerned with the influence of diet on the serum proteins with greatest emphasis on the quality and quantity of protein.^{3,4,5} Notwithstanding the universal employment of the albino rat in nutritional investigations, the majority of the serum protein studies have used man or the dog as subjects^{3,4,5} and there is some evidence of species' differences in the responses observed.³ In a few experiments in which electrophoretic analyses were carried out on rat serum, pooled samples were employed.^{6,7}

The presence in the serum of man and animals of protein-bound carbohydrates in amounts several times greater than that of free glucose has been known for many years. In contrast with the large number of investigations on the effects of nutritional states on blood glucose and serum protein levels, few studies have been concerned with the influence of diet on the carbohydrate-containing proteins of serum. The effects of fasting and the feeding of carbohydrate, protein, or fat test-meals on the serum glycoproteins of normal individuals have been studied clinically. The reported results were negative and have been recently reviewed.^{8,9} Experimental investigations have demonstrated that inanition^{10,11} and protein depletion¹¹ cause decreases in both the proteins and protein-bound carbohydrates of rat serum.

Inasmuch as protein depletion produced significant decreases in the concentrations of the serum components,¹¹ modifications of the depletion-repletion technique of Cannon and his associates¹² have been employed to investigate the effects of dietary protein on the serum proteins and glycoproteins of adult, male rats. Included are data on groups of animals depleted as a result of tumor growth with concomitant caloric restriction due to anorexia.

The development of chemical methods that require smaller volumes of serum has greatly facilitated and increased the number of determinations that may be carried out on a single serum sample from a small experimental animal. To avoid the divergent effects of multiple hemorrhages,¹³ each rat was bled once. Blood samples were obtained by cardiac puncture under ether anesthesia. Adult male Sprague-Dawley rats were employed in all studies. The use of larger animals was dictated by the difficulties inherent in obtaining adequate blood samples from smaller rats, especially when depleted.

The rats were housed singly in suspended-type wire cages with wire bottoms.

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They were maintained on an antibiotic-free commercial chow* and tap water, both supplied *ad libitum*. Hematologic, chemical and electrophoretic analyses were carried out by methods previously reported.^{14,15} Analysis of the data was made by standard statistical procedures employing the *t* test.¹⁶

Effects of the Degree of Protein Depletion and Repletion on the Serum Proteins and Glycoproteins

An earlier study¹¹ had demonstrated that feeding a protein-free diet until a 25 per cent weight loss occurred caused significant decreases in serum protein and glycoprotein concentrations. The following study was initiated to investigate the effects of different degrees of protein depletion and repletion on the serum components.

Depletion. The rats were kept on the following protein-free diet.†

	Per cent	Vitamin supplement	mg./kg.
Cornstarch	70	alpha tocopherol	110
Alphacel (cellulose)	15	ascorbic acid	1,000
Vegetable oil	10	inositol	110
Salt mixture (U.S.P. XIV)	4	choline chloride	1,660
Cod-liver oil	1	menadione	55
		para-aminobenzoic acid	110
		niacin	100
		riboflavin	22
		thiamine hydrochloride	22
		calcium pantothenate	67
		folic acid	2
		biotin	0.44
		Vitamin B ₁₂	0.03
		Vitamin A 20,000 U./kg.	
		Vitamin D 2,220 U./kg.	

Groups of rats were sacrificed after losing 10, 25, 33, and 40 per cent of their original weight.

Repletion. A commercial chow‡ with a protein content of approximately 25 per cent was fed *ad libitum* to animals that had sustained a 25 per cent loss of weight on the protein-free diet. Groups of rats were sacrificed after regaining 35, 70, and 100 per cent of their lost weight. In addition, one group was allowed to survive 4 weeks following weight repletion.

Controls. Two normal groups were employed. Group A1 served as a base line control and A2 as an age and weight control. Group A3 was included to determine the effects of prior depletion and repletion on 400-gm. rats. Electrophoretic analyses were not carried out on serum samples from this group.

Electrophoretic studies. Body weight curves during protein depletion and repletion are presented in FIGURE 1. More detailed information is included in TABLE 1. The weight curves were characterized by marked inflections following dietary change. Alterations in plasma volume as indicated by hematocrit values were in general agreement with the earlier studies reviewed by Keys *et al.*³ and by Allison.⁴ In the early stages of protein depletion (B1) there was marked hemoconcentration that was followed by hemodilution as depletion

* Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

† Obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡ Purina Laboratory Chow.

progressed. Significant hemodilution occurred during repletion. Following repletion (A3, C4), hematocrit levels were in the normal range. Hemoglobin was restored more slowly than the serum proteins (TABLE 2).

Results of electrophoretic analyses are summarized in FIGURES 2a, b, and c. Feeding a protein-free diet for one week (B1) caused highly significant decreases in total protein, albumin, and β -globulin concentrations. A weight loss of 25 per cent (B2) elicited significant decreases in total protein, albumin,

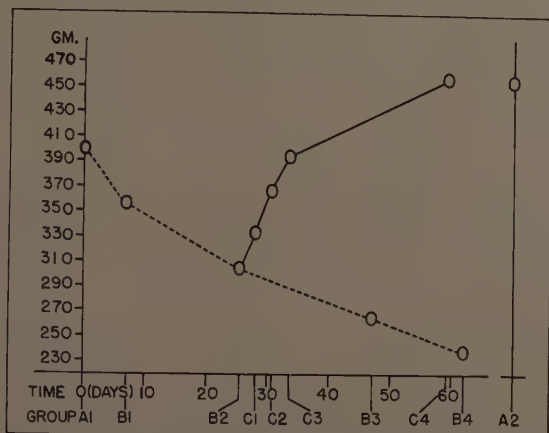


FIGURE 1. Effects of protein depletion and repletion on body weight. Dashed and solid lines indicate when protein-free and stock diets respectively were fed. Reproduced by permission of the Proceedings of the Society for Experimental Biology and Medicine.

TABLE 1*
GENERAL AND HEMATOLOGIC DATA

Group	No. rats	Initial wt.† (gm.)	Depletion wt.† (gm.)	Depletion time† (days)	Final wt.† (gm.)	Repletion time (days)	Hemoglobin† (gm. %)	Hematocrit† (%)
A1. Normal (400 gm.)	40	404±3.0					14.3±0.19	45±0.3
A2. Normal (460 gm.)	14	457±2.9‡					13.8±0.20§	46±0.5
A3. Repletion control	15	327±2.5‡	247±2.0‡	29.9	399±4.0	26.1	13.6±0.22‡	45±0.3
B1. 10% depleted	13	400±4.3	357±3.9‡	7.0			14.7±0.15	49±0.3‡
B2. 25% depleted	26	399±3.2	301±2.9‡	26.2			13.5±0.28‡	43±0.4‡
B3. 33% depleted	9	401±6.5	266±4.0‡	46.8			11.4±0.36‡	36±1.4‡
B4. 40% depleted	10	402±5.4	239±3.4‡	62.0			11.8±0.33‡	42±1.7‡
C1. 35% repleted	12	397±5.5	299±4.0‡	27.2	333±4.2‡	2.5	11.8±0.24‡	38±0.6‡
C2. 70% repleted	13	399±6.4	297±5.1‡	27.2	368±5.9‡	5.0	11.8±0.30‡	37±0.5‡
C3. 100% repleted	28	403±3.8	303±2.7‡	21.5	402±4.0	8.6	12.4±0.17‡	40±0.5‡
C4. 4-wk. postrepletion	26	401±3.1	300±2.5‡	24.2	459±4.0‡	33.8	14.0±0.20	45±0.5

* Part of data from Weimer *et al.*¹⁷

† Including the standard error of the mean.

‡ Statistically significant differences from normal values (A1) are indicated as follows:

‡ P = <0.01.

§ P = <0.05 > 0.01.

α_1 -, and α_2 -globulin fractions concomitant with a significant increase in γ -globulin levels. Further depletion was characterized by divergent responses in the serum fractions. Of considerable interest was the high A/G ratio in the 40 per cent depleted group (B4), reflecting the increase in the albumin fraction in severe depletion. A similar increase in the ratio has been observed for tumor-bearing rats in the terminal stages of the disease (FIGURE 3a).

Total serum protein values, although not reflecting the alterations in the subfractions, were remarkably stable following 25 per cent depletion. The reports of Steinbock and Tarver¹⁸ and Jeffay and Winzler¹⁹ suggest that this was a result of a slower rate of turnover. At the end of the depletion phase (B4), the concentrations of all fractions were significantly less than normal.

TABLE 2*
SUMMARY OF SERUM PROTEIN DETERMINATIONS DURING
PROTEIN DEPLETION AND REPLETION

Group	Total serum protein (gm. %)	Seromucoid protein (gm. %)	Albumin protein (gm. %)	Globulin protein (gm. %)
A1. Normal (400 gm.)	5.8 \pm 0.04	0.26 \pm 0.005	2.6 \pm 0.03	3.2 \pm 0.04
A2. Normal (460 gm.)	6.1 \pm 0.09†	0.29 \pm 0.012†	2.6 \pm 0.04	3.5 \pm 0.07†
A3. Repletion control	6.1 \pm 0.04†	0.22 \pm 0.006†	2.6 \pm 0.04	3.5 \pm 0.05†
B1. 10% depleted	5.3 \pm 0.05†	0.24 \pm 0.007†	2.4 \pm 0.05†	2.9 \pm 0.06†
B2. 25% depleted	4.8 \pm 0.05†	0.23 \pm 0.008†	2.3 \pm 0.04†	2.5 \pm 0.05†
B3. 33% depleted	4.7 \pm 0.06†	0.26 \pm 0.016	ND	ND
B4. 40% depleted	4.7 \pm 0.10†	0.29 \pm 0.015†	2.5 \pm 0.07	2.2 \pm 0.07†
C1. 35% repleted	5.5 \pm 0.09†	0.26 \pm 0.014	2.5 \pm 0.05	3.0 \pm 0.08†
C2. 70% repleted	5.9 \pm 0.07	0.21 \pm 0.007†	2.7 \pm 0.07	3.2 \pm 0.07
C3. 100% repleted	6.0 \pm 0.05†	0.23 \pm 0.007†	2.6 \pm 0.04	3.4 \pm 0.04†
C4. 4-wk. postrepletion	6.0 \pm 0.04†	0.28 \pm 0.005	2.3 \pm 0.05†	3.7 \pm 0.07†

* Part of data from Weimer *et al.*¹⁷ The data include standard error of the mean.

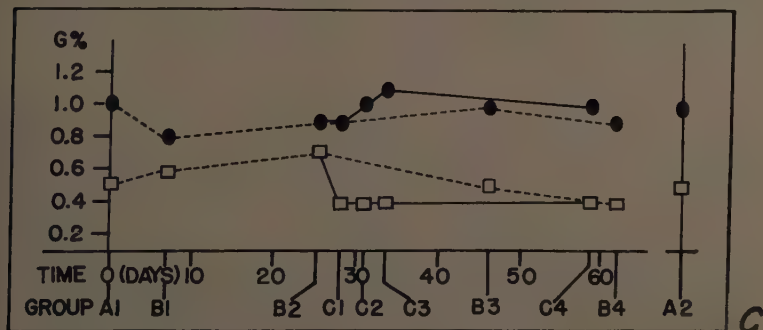
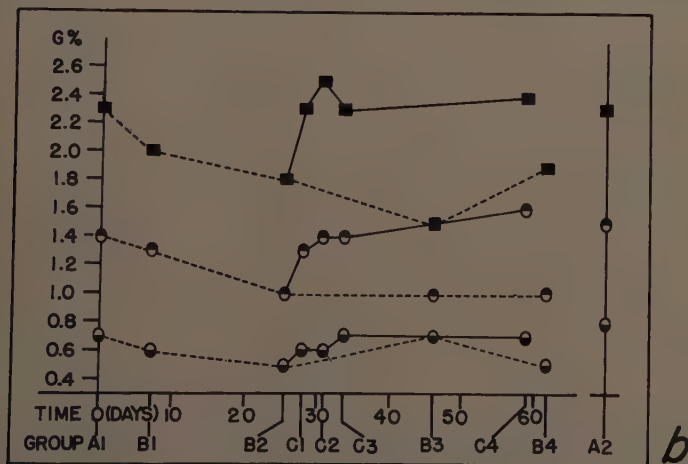
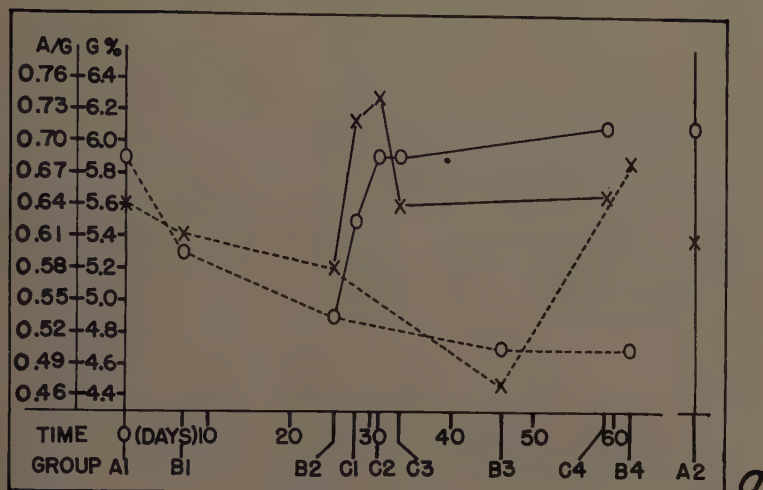
Statistically significant differences from normal values (C4 was compared with A2; other groups with A1) are indicated as follows:

† P = <0.01.

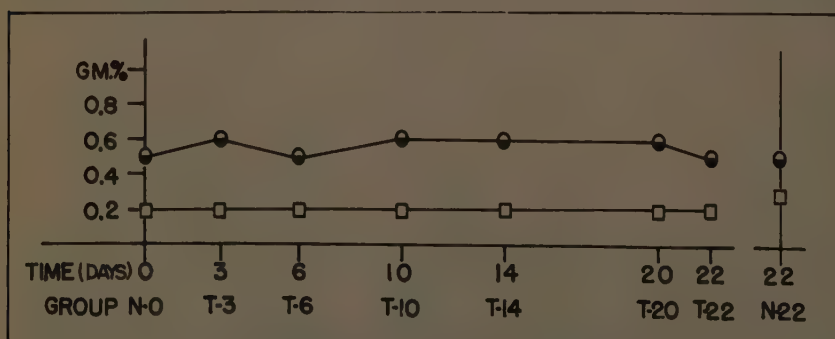
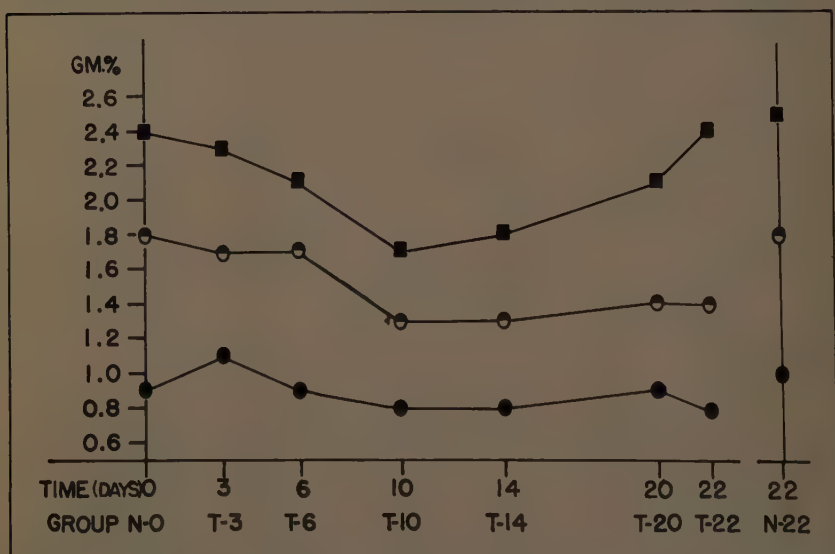
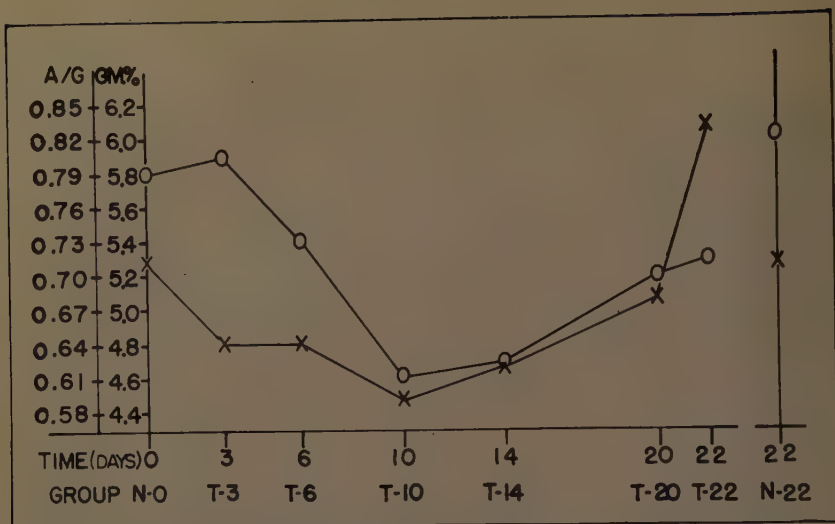
‡ P = <0.05 > 0.01.

Rapid increases occurred in the major serum components upon introduction of the stock diet (C1). Total protein, albumin, α_1 -, α_2 - and β -globulin concentrations were restored to normal before weight repletion was achieved (C2). The γ -globulin fraction deviated from the general pattern of response during both depletion and repletion. Comparison of the two normal groups (A1, A2) showed no significant differences in the distribution of the serum proteins with increased age and weight, although the total serum protein concentration of the older group was significantly greater.

Glycoprotein studies. My associates and I have had little success in studying changes in the glycoproteins of rat serum by the periodic acid-Schiffs stain following filter paper electrophoresis. Approximately 65 per cent of the PAS-positive material in the serum of Sprague-Dawley rats is in the α_1 -globulin fraction. We have therefore employed chemical separations for investigating the partition of the protein-bound carbohydrates of serum. In these studies, the protein-bound hexose and protein concentrations of whole serum and of the seromucoid, albumin and globulin fractions have been determined. The



FIGURES 2a, b, and c. Effects of protein depletion and repletion on serum protein concentrations. Dashed and solid lines indicate when protein-free and stock diets respectively were fed. Key: \bigcirc = total serum protein; \times = A/G ratio; \blacksquare = albumin; \bullet = α_1 globulin; \circ = α_2 globulin; \bullet = β -globulin; \square = γ -globulin. Reproduced by permission of the Proceedings of the Society for Experimental Biology and Medicine.



FIGURES 3a, b, and c. Effects of tumor growth on serum protein concentrations. Key: O, total protein; X, A/G ratio; ■, albumin; ●, α_1 globulin; ●, β globulin; ●, α_2 globulin; □, γ globulin.

albumin and globulin fractions were separated by a modified Pillemer-Hutchinson procedure.¹³

Protein values for the chemically separated globulin fraction were somewhat lower than those obtained by electrophoresis, while the corresponding albumin concentrations were higher (TABLE 2). The curves for the albumin and globulin fractions determined by the two different procedures roughly paralleled each other with the exception of the results for the C4 group. Although some changes of statistical significance occurred in seromuroid protein levels, it was apparent that the fraction was affected to only a relatively small extent by the dietary regimens. Elevated globulin concentrations were found at repletion (C3), postrepletion (A3, C4) and with increased age and weight (A2).

TABLE 3*
SUMMARY OF SERUM PROTEIN-BOUND CARBOHYDRATE DETERMINATIONS
DURING PROTEIN DEPLETION AND REPLETION

Group	Total serum glycoprotein (mg. %)	Seromuroid hexose (mg. %)	Albumin hexose (mg. %)	Globulin hexose (mg. %)
A1. Normal (400 gm.)	167 ± 1.0	24 ± 0.6	18 ± 0.7	149 ± 1.2
A2. Normal (460 gm.)	181 ± 3.0†	25 ± 0.7	16 ± 1.2	165 ± 3.1†
A3. Repletion control	167 ± 1.8	18 ± 0.7†	20 ± 1.3	147 ± 2.4
B1. 10% depleted	146 ± 1.9†	22 ± 0.6	21 ± 1.1†	125 ± 1.9†
B2. 25% depleted	124 ± 2.2†	20 ± 0.6†	16 ± 0.6†	108 ± 2.4†
B3. 33% depleted	131 ± 2.5†	23 ± 1.4	ND	ND
B4. 40% depleted	122 ± 3.0†	24 ± 1.3	18 ± 1.5	104 ± 3.1†
C1. 35% repleted	130 ± 2.0†	21 ± 1.0†	15 ± 1.2†	115 ± 2.3†
C2. 70% repleted	139 ± 2.6†	18 ± 0.7†	15 ± 1.3†	124 ± 2.7†
C3. 100% repleted	155 ± 1.3†	21 ± 0.5†	14 ± 0.9†	141 ± 1.4†
C4. 4-wk. postrepletion	188 ± 1.6†	24 ± 0.5	12 ± 0.9†	176 ± 1.4†

* Part of data from Weimer *et al.*¹⁷ The data include standard error of the mean.

Significant differences from normal values (C4 was compared with A2; other groups with A1) are indicated as follows:

† P = <0.01.

‡ P = <0.05 > 0.01.

Results of protein-bound carbohydrate analyses are presented in TABLE 3. Total serum glycoprotein levels declined rapidly during the early stages of depletion, primarily as a consequence of the decreases in globulin polysaccharide. Protein-bound carbohydrate values became stabilized after 25 per cent depletion (B2), with the exception of seromuroid hexose which, like the protein component of the fraction, increased (TABLE 2).

Introduction of the stock diet caused significant increases in total serum glycoprotein and globulin polysaccharide concentrations over depletion levels. However, at repletion, all protein-bound carbohydrate values were significantly less than normal. In the postrepletion period (C4), total serum glycoprotein concentrations became significantly elevated, due to the increase in the seromuroid and globulin fractions. Values for the repletion control group (A3) were in the normal range, with the exception of seromuroid hexose.

To compensate for fluctuations in plasma volume and further to define their differential response, a study was made of the relationships of the protein-bound carbohydrates and proteins of serum (TABLE 4). The most pronounced

early change during depletion was the significant decrease in the ratio of the globulin fraction (B1). In severe depletion (B4), however, the ratio for the fraction was restored to normal, notwithstanding a significant decrease in the ratio for total serum.

A general decrease in the ratios occurred in the early stage of repletion (C1). At repletion (C3) only the ratio for the seromucoid fraction was in the normal range. Ratios for the postdepletion group (C4) were in the normal range when compared with their weight control group (A2). It was apparent that in the four-week period following weight repletion, the protein-bound carbohydrates of the globulin fraction had increased to a greater extent than the protein components. Ratios for the repletion control group (A3) were significantly less than normal for total serum and the seromucoid and globulin fractions. This

TABLE 4*
PROTEIN-BOUND CARBOHYDRATE/PROTEIN RATIOS†
DURING PROTEIN DEPLETION AND REPLETION

Group	Total serum (%)	Seromucoid fraction (%)	Albumin fraction (%)	Globulin fraction (%)
A1. Normal (400 gm.)	2.9 ± 0.03	9.2 ± 0.19	0.7 ± 0.03	4.7 ± 0.08
A2. Normal (460 gm.)	3.0 ± 0.04	8.6 ± 0.29	0.6 ± 0.05	4.7 ± 0.08
A3. Repletion control	2.7 ± 0.03‡	8.2 ± 0.12‡	0.8 ± 0.05	4.2 ± 0.03‡
B1. 10% depleted	2.8 ± 0.04	9.2 ± 0.15	0.9 ± 0.05‡	4.3 ± 0.04‡
B2. 25% depleted	2.6 ± 0.04‡	8.7 ± 0.10§	0.7 ± 0.02	4.3 ± 0.11‡
B3. 33% depleted	2.8 ± 0.04	8.8 ± 0.19	ND	ND
B4. 40% depleted	2.6 ± 0.12‡	8.3 ± 0.24§	0.7 ± 0.05	4.7 ± 0.12
C1. 35% repleted	2.4 ± 0.04‡	8.1 ± 0.51§	0.6 ± 0.05	3.8 ± 0.07‡
C2. 70% repleted	2.4 ± 0.04‡	8.6 ± 0.18	0.6 ± 0.06	3.9 ± 0.08‡
C3. 100% repleted	2.6 ± 0.02‡	9.1 ± 0.24	0.5 ± 0.04‡	4.1 ± 0.04‡
C4. 4-wk. postrepletion	3.1 ± 0.03	8.6 ± 0.11	0.5 ± 0.05	4.8 ± 0.03

* Part of data from Weimer *et al.*¹⁷ The data include standard error of the mean.

† Protein-bound hexose ÷ protein × 100.

‡ Statistically significant differences from normal values (C4 was compared with A2; other groups with A1) are indicated as follows:

‡ P = <0.01.

§ P = <0.05 > 0.01.

also may reflect a preferential utilization of glycoproteins during the period of more rapid postdepletion growth characteristic of younger rats. This group (A3) gained an average of 5.8 gm. per day contrasted with approximately 1.8 gm. per day for the older animals of group C4.

Influence of the Dietary Levels of Protein and Carbohydrate on the Serum Proteins and Glycoproteins

As previously noted, a protein-free diet caused significant decreases in the serum proteins and glycoproteins. In view of these results, it seemed probable that employment of the depletion-repletion technique of Cannon and associates^{12,20} would provide a convenient method for evaluating the effects of different levels of dietary protein and carbohydrate on the serum constituents. The method, as Cannon has emphasized,²⁰ utilizes a basic principle common to many bioassays, that is, the production of a deficit followed by a measurement of the replacement of the deficit.

Depletion. Groups of rats were depleted by inanition, and the feeding of protein-free or 2 per cent protein diets.

Repletion. The animals were repleted after a 25 per cent weight loss by feeding diets varying in protein and carbohydrate content.

Diets. Composition of the purified diets is presented in TABLE 5. In addi-

TABLE 5
COMPOSITION OF PURIFIED DIETS

Diet	Casein (%)	Cornstarch (%)	Vegetable oil (%)	Salt mixture (U.S.P. XIV) (%)	Alphacel (cellulose) (%)	Cod-liver oil (%)	Vitamin supplement
Depletion							
Protein-free	—	70	10	4	15	1	+
2% protein	2	69	10	4	14	1	+
Repletion							
8% protein	8	78	10	4	—	—	+
17% protein	17	69	10	4	—	—	+
27% protein	27	59	10	4	—	—	+
40% protein	40	46	10	4	—	—	+
64% protein	64	22	10	4	—	—	+

TABLE 6*
GENERAL AND HEMATOLOGIC DATA

Group	No. rats	Original weight (gm.)	Depleted weight (gm.)	Repleted weight (gm.)	Hemoglobin (gm. %)	Hematocrit (%)
A. Normal	40	404 ± 3.0			14.3 ± 0.19	45 ± 0.3
B. Inanition	20	407 ± 8.5	305 ± 6.2†		16.5 ± 0.28†	53 ± 0.8†
C. Acute depletion	26	399 ± 3.2	301 ± 2.9†		13.5 ± 0.28†	43 ± 0.4†
D. Chronic depletion	18	399 ± 4.8	301 ± 3.4†		12.3 ± 0.37†	42 ± 1.2†
E1. 8% protein	19	403 ± 5.8	306 ± 4.5†	400 ± 6.3	13.1 ± 0.09†	43 ± 0.5†
E2. 17% protein	27	399 ± 4.6	303 ± 3.3†	402 ± 4.2	12.3 ± 0.31†	42 ± 0.7†
E3. 27% protein	19	403 ± 6.6	302 ± 4.8†	406 ± 4.7	13.3 ± 0.20†	42 ± 0.6†
E4. 40% protein	18	405 ± 3.6	303 ± 3.3†	400 ± 3.9	12.1 ± 0.13†	40 ± 0.4†
E5. 64% protein	17	406 ± 2.6	309 ± 2.2†	406 ± 3.3	13.1 ± 0.21†	43 ± 0.5†
F1. Stock diet	15	407 ± 3.3	308 ± 2.8†	408 ± 3.5	12.8 ± 0.23†	41 ± 0.6†
F2. Stock diet	18	401 ± 4.3	301 ± 2.6†	402 ± 4.4	12.0 ± 0.23†	40 ± 0.7†

* Data from Weimer and Nishihara.^{11,14,21} Results include standard error of the mean.

† Significant differences from normal values are indicated as follows:

† P = <0.01.

tion, ground Purina Laboratory Chow with a protein content of 25 per cent and a nitrogen free extract value of 47.5 per cent was also employed in the repletion of two groups (F1, F2).

General and hematologic data are presented in TABLE 6. Inanition produced hemoconcentration as indicated by significantly increased hematocrit and hemoglobin values. Other dietary regimens cause varying degrees of hemodilution and decreased hemoglobin concentrations.

Nutritional data are summarized in TABLE 7. Protein was most effectively

utilized in the 8 per cent casein diet. The 40 per cent casein and the ground stock diets were the best balanced for weight repletion as shown by rate of weight gain. Utilization of the complete diet was best in the 40 per cent casein group as indicated by the food efficiency ratio. Of interest were the observed differences between the two groups repleted on the stock diet (F1, F2) with respect to repletion time and food and protein efficiency ratios. Although the weight loss in each group was identical, the chronically depleted group (F2) did not respond as rapidly or as effectively to an adequate diet.

TABLE 7*
SUMMARY OF NUTRITIONAL DATA†

Group	Diet	Depletion time (days)	Repletion time (days)	Food consumption (gm.)	Weight gained (gm.)	Food efficiency ratio‡	Protein consumption (gm.)	Protein efficiency ratio§
A. Normal	Stock							
B. Inanition	—	7.9						
C. Acute depletion	Protein-free	19.9						
D. Chronic depletion	2% protein	61.8						
E1. 8% protein	Protein-free: 8% casein	21.1	16.8	396.5	94	0.24	31.7	3.0
E2. 17% protein	Protein-free: 17% casein	20.6	11.2	291.6	99	0.34	49.6	2.0
E3. 27% protein	Protein-free: 27% casein	21.0	11.5	291.0	104	0.36	78.6	1.3
E4. 40% protein	Protein-free: 40% casein	16.1	8.6	191.8	97	0.51	76.7	1.3
E5. 64% protein	Protein-free: 64% casein	17.0	11.9	235.6	97	0.41	150.8	0.6
F1. Stock	Protein-free: ground stock	16.8	8.7	233.8	100	0.42	59.5	1.7
F2. Stock	2% protein: ground stock	58.0	11.9	309.6	101	0.33	77.3	1.3

* Data from Weimer and Nishihara.^{11,14,21}

† Average values.

‡ Food efficiency ratio = weight gained/food consumed.

§ Protein efficiency ratio = weight gained/protein consumed.

The effects of the dietary regimens on the partition of serum proteins as determined by electrophoretic analyses are presented in TABLE 8. All depletion regimens caused significant decreases in the albumin, α_1 - and α_2 -globulin fractions. Starvation and acute depletion also elicited significant decreases in the concentrations of β -globulin concurrent with increases in the γ -globulin fraction. All depletion diets, however, had the same relative influence on the loss of albumin and the serum globulins as shown by the similarity of the A/G ratios.

Repletion with the 8 per cent protein diet following acute depletion restored the albumin, α_2 - and β -globulin fractions to normal. A higher level of dietary protein (17 per cent) was required to restore the α_1 globulins. With the exception of the 8 per cent protein, all other purified diets were not only adequate to restore total serum protein levels to normal, but elicited substantial in-

TABLE 8*
EFFECTS OF PROTEIN DEPLETION AND REPLETION ON THE PARTITION OF SERUM PROTEINS IN THE ADULT RAT

Group	Total serum protein (gm. %)	Albumin (gm. %)	Globulin				A/G ratio
			α_1 (gm. %)	α_2 (gm. %)	β (gm. %)	γ (gm. %)	
A. Normal	5.8 \pm 0.04	2.0 \pm 0.03	1.5 \pm 0.03	0.7 \pm 0.03	1.1 \pm 0.03	0.5 \pm 0.02	0.53 \pm 0.014
B. Depleted							
Inanition	5.1 \pm 0.02†	1.7 \pm 0.05†	1.3 \pm 0.04†	0.5 \pm 0.03†	0.9 \pm 0.03†	0.7 \pm 0.03†	0.50 \pm 0.017
C. Protein-free	4.8 \pm 0.05†	1.7 \pm 0.02†	1.2 \pm 0.03†	0.5 \pm 0.02†	0.8 \pm 0.02†	0.6 \pm 0.03†	0.53 \pm 0.010
D. 2% protein	4.9 \pm 0.07†	1.7 \pm 0.04†	1.3 \pm 0.03†	0.4 \pm 0.02†	1.0 \pm 0.05	0.5 \pm 0.04	0.53 \pm 0.015
Repleted							
E1. 8% protein	5.5 \pm 0.07†	2.0 \pm 0.05	1.3 \pm 0.07†	0.6 \pm 0.03	1.1 \pm 0.04	0.5 \pm 0.02	0.57 \pm 0.021
E2. 17% protein	6.4 \pm 0.08†	2.2 \pm 0.04†	1.5 \pm 0.06	0.8 \pm 0.04	1.3 \pm 0.05†	0.6 \pm 0.05	0.55 \pm 0.018
E3. 27% protein	6.0 \pm 0.06†	2.0 \pm 0.06	1.7 \pm 0.05†	0.7 \pm 0.04	1.1 \pm 0.04	0.5 \pm 0.03	0.50 \pm 0.023
E4. 40% protein	6.2 \pm 0.08†	2.2 \pm 0.06†	1.6 \pm 0.06	0.7 \pm 0.04	1.2 \pm 0.03	0.5 \pm 0.04	0.55 \pm 0.020
E5. 64% protein	6.1 \pm 0.07†	2.1 \pm 0.07	1.5 \pm 0.05	0.8 \pm 0.04	1.2 \pm 0.03	0.5 \pm 0.05	0.52 \pm 0.029
F1. Stock	6.1 \pm 0.06†	2.3 \pm 0.06†	1.5 \pm 0.04	0.7 \pm 0.04	1.1 \pm 0.04	0.5 \pm 0.04	0.61 \pm 0.030†
F2. Stock	5.8 \pm 0.07	2.3 \pm 0.06†	1.5 \pm 0.06	0.5 \pm 0.04†	1.1 \pm 0.05	0.4 \pm 0.03†	0.66 \pm 0.071†

* Data from Weimer, Bell *et al.*²² The findings include the standard error of the mean.

† Statistically significant differences from normal values are indicated as follows:

† P = <0.01.

† P = <0.05 > 0.01.

creases. The 17 per cent protein diet was the best balanced for the restoration of the serum proteins as measured by maximum increase of serum values over depletion levels. The comparative effects of acute and chronic depletion on capacity to respond to a standard diet are shown by the results of groups F1 and F2. Although the weight loss in each group was identical, the chronically depleted rat was unable to respond to the same extent during repletion. The major divergences were in α_2 globulin and total serum protein values. The increased A/G ratios for the groups F1 and F2 suggested that repletion on a diet of natural products²³ promoted the regeneration of serum albumin to a greater extent than a purified diet of similar protein content, E3.

The current studies have demonstrated that in the rat, the components of the globulin fraction are more affected by protein depletion than the correspond-

TABLE 9*
EFFECTS OF PROTEIN DEPLETION AND REPLETION ON
CHEMICALLY-SEPARATED PROTEIN FRACTIONS

Group	Total serum protein (gm. %)	Seromucoid protein (gm. %)	Albumin protein (gm. %)	Globulin protein (gm. %)	A/G ratio
A. Normal	5.8±0.04	0.26±0.005	2.6±0.03	3.2±0.04	0.81±0.018
B. Depletion	5.1±0.02†	0.23±0.004†	2.6±0.02	2.5±0.02†	1.04±0.041†
C. Inanition	4.8±0.05†	0.23±0.008†	2.3±0.04†	2.5±0.05†	0.92±0.033
D. Acute depletion	4.9±0.07†	0.21±0.008†	2.4±0.04†	2.5±0.05†	0.96±0.018†
E. Chronic depletion	4.9±0.07†	0.21±0.008†	2.4±0.04†	2.5±0.05†	0.96±0.018†
F. Repletion	4.9±0.07†	0.21±0.008†	2.4±0.04†	2.5±0.05†	0.96±0.018†
E1. 8% protein	5.5±0.07†	0.19±0.004†	2.7±0.06	2.8±0.07†	0.96±0.042†
E2. 17% protein	6.4±0.08†	0.21±0.006†	2.8±0.07†	3.6±0.07†	0.78±0.027
E3. 27% protein	6.0±0.06†	0.26±0.005	2.6±0.05	3.4±0.06†	0.76±0.023
E4. 40% protein	6.2±0.08†	0.24±0.007	2.7±0.05	3.5±0.08†	0.77±0.025
E5. 64% protein	6.1±0.07†	0.26±0.007	2.6±0.05	3.5±0.05†	0.74±0.018
F1. Stock	6.1±0.06†	0.25±0.010	2.6±0.07	3.5±0.06†	0.74±0.037
F2. Stock	5.8±0.07	0.19±0.007†	2.5±0.06	3.3±0.07	0.76±0.029

* Data from Weimer and Nishihara.^{11,14,21} The findings include the standard error of the mean. Significant differences from normal values are indicated as follows:

† P = <0.01.

‡ P = <0.05 > 0.01.

ing fractions of man and the dog.^{3,4,5} Diets with a protein content of 17 per cent were found to be adequate for the restoration of the serum proteins in the adult, male rat following acute depletion.

The effects of the various depletion and repletion diets on the chemically-separated protein fractions are presented in TABLE 9. With the notable exception of the albumin value for the inanition group (B), the concentrations of all other fractions were greatly decreased during depletion. The result for the albumin fraction of Group B is not in agreement with the electrophoretic data (TABLE 8). With the exception of the 8 per cent level of protein, all other repletion diets were adequate for the restoration of the albumin and globulin fractions. A higher level of dietary protein (27 per cent) was required to restore the protein moiety of the seromucoid fraction to normal. In the group previously subjected to chronic depletion, F2, the concentration of the fraction was conspicuously low.

Data on protein-bound carbohydrates of serum are summarized in TABLE 10. With the exception of the albumin fraction, highly significant decreases occurred in all other components during depletion. Following weight repletion on the 8 per cent protein diet, elevation of globulin hexose led to an increase in total serum glycoprotein. The values, however, were significantly lower than normal. A decrease over the depletion value (Group C) was observed for seromucoid hexose. Restoration of all the protein-bound carbohydrates of serum was not achieved until the protein content of the diet was 27 per cent. Repletion with the 40 and 64 per cent protein diets elicited changes similar to those observed for the 27 per cent group. Certain quantitative differences were noted between the groups repleted with the ground stock diet (F1, F2) and the group

TABLE 10*
EFFECTS OF PROTEIN DEPLETION AND REPLETION ON THE
PROTEIN-BOUND CARBOHYDRATES OF SERUM

Group	Total serum glycoprotein (mg. %)	Seromucoid hexose (mg. %)	Albumin hexose (mg. %)	Globulin hexose (mg. %)	A/G ratio
A. Normal Depletion	167 ± 1.0	24 ± 0.6	18 ± 0.7	149 ± 1.2	0.12 ± 0.005
B. Inanition	132 ± 1.7†	21 ± 0.6‡	16 ± 1.4	116 ± 2.3†	0.14 ± 0.015
C. Acute depletion	124 ± 2.2†	20 ± 0.6†	16 ± 0.6‡	108 ± 2.4†	0.15 ± 0.007
D. Chronic depletion	124 ± 1.7†	18 ± 0.9†	18 ± 1.2	106 ± 1.8†	0.17 ± 0.012†
Repletion					
E1. 8% protein	135 ± 1.8†	16 ± 0.5†	16 ± 1.0	119 ± 2.1†	0.13 ± 0.011
E2. 17% protein	162 ± 1.9	16 ± 0.5†	21 ± 1.2	141 ± 2.0‡	0.15 ± 0.012†
E3. 27% protein	162 ± 2.3	23 ± 0.8	15 ± 1.6	147 ± 1.8	0.10 ± 0.011†
E4. 40% protein	161 ± 2.7	22 ± 0.6	15 ± 1.3	146 ± 2.5	0.10 ± 0.009†
E5. 64% protein	163 ± 2.6	23 ± 0.4	14 ± 1.3†	149 ± 2.3	0.09 ± 0.008†
F1. Stock	157 ± 1.6†	22 ± 0.5	14 ± 1.4†	143 ± 1.7†	0.10 ± 0.010
F2. Stock	153 ± 2.2†	17 ± 0.5†	17 ± 1.4	136 ± 2.5†	0.13 ± 0.011

* Data from Weimer and Nishihara.^{11,14,21} The findings include the standard error of the mean.

† Significant differences from normal values are indicated as follows:

† P = <0.01.

‡ P = <0.05 > 0.01.

(E3) repleted on a purified diet of comparable protein content. Thus the carbohydrate-containing proteins of serum were most sensitive to alterations in the protein content of the diet in the range from zero to 27 per cent. However, increased dietary protein levels had negligible additional effects on either the protein-bound carbohydrates or proteins of serum.

Certain deductions may also be made with regard to the influence of dietary carbohydrate on the concentrations of the serum glycoproteins. The amount of starch in the purified repletion diets varied from 22 per cent (Group E5) to 78 per cent (Group E1). In diets adequate for the restoration of carcass weight, it was apparent that the concentration of the protein-bound carbohydrates of serum did not parallel the level of starch in the diet. The results strongly suggest that serum glycoproteins play no role in the transport of dietary carbohydrate. It may also be inferred from the data obtained in the

serial study (TABLES 3 and 4) that the level of carbohydrate in the diet was not a limiting factor.

In contrast to the results for total serum glycoprotein and globulin hexose, the corresponding protein components were not only restored to normal, but were significantly increased in all but two repletion groups. Groups E1 (8 per cent casein) and F2, which had been previously subjected to chronic depletion, were the exceptions. In an attempt to delineate further the differential responses of the proteins and protein-bound carbohydrates of serum to the various depletion and repletion regimens, a study was made of their relationships (TABLE 11). Decreased ratios for total serum occurred in all experimental groups and with the exception of Group B (Inanition), the same results were

TABLE 11*
PROTEIN-BOUND CARBOHYDRATE/PROTEIN RATIOS
DURING PROTEIN DEPLETION AND REPLETION

Group	Protein-bound carbohydrate/protein $\times 100$			
	Total serum (%)	Seromucoid fraction (%)	Albumin fraction (%)	Globulin fraction (%)
A. Normal	2.9 ± 0.03	9.2 ± 0.19	0.7 ± 0.03	4.7 ± 0.08
Depletion				
B. Inanition	$2.6 \pm 0.02^\dagger$	9.1 ± 0.21	0.6 ± 0.03	4.6 ± 0.05
C. Acute depletion	$2.6 \pm 0.04^\dagger$	$8.7 \pm 0.10^\dagger$	0.7 ± 0.02	$4.3 \pm 0.11^\dagger$
D. Chronic depletion	$2.5 \pm 0.03^\dagger$	$8.6 \pm 0.10^\dagger$	0.8 ± 0.05	$4.2 \pm 0.08^\dagger$
Repletion				
E1. 8% protein	$2.5 \pm 0.03^\dagger$	$8.4 \pm 0.15^\dagger$	0.6 ± 0.04	$4.3 \pm 0.07^\dagger$
E2. 17% protein	$2.5 \pm 0.02^\dagger$	$7.7 \pm 0.18^\dagger$	0.8 ± 0.04	$3.9 \pm 0.06^\dagger$
E3. 27% protein	$2.7 \pm 0.02^\dagger$	8.8 ± 0.28	0.6 ± 0.06	$4.3 \pm 0.05^\dagger$
E4. 40% protein	$2.6 \pm 0.03^\dagger$	9.2 ± 0.30	0.6 ± 0.05	$4.2 \pm 0.05^\dagger$
E5. 64% protein	$2.7 \pm 0.04^\dagger$	8.8 ± 0.24	0.6 ± 0.05	$4.3 \pm 0.05^\dagger$
F1. Stock	$2.6 \pm 0.03^\dagger$	9.0 ± 0.38	$0.5 \pm 0.06^\dagger$	$4.1 \pm 0.06^\dagger$
F2. Stock	$2.6 \pm 0.04^\dagger$	9.0 ± 0.18	0.7 ± 0.05	$4.1 \pm 0.06^\dagger$

* Data from Weimer and Nishihara.^{11,14,21} The findings include the standard error of the mean.

Significant differences from normal values are indicated as follows:

$^\dagger P = <0.01$.

$^\ddagger P = <0.05 >0.01$.

observed for the globulin fraction. The ratios for the carbohydrate-poor albumin fraction were consistently normal irrespective of the nutritional state. No consistent pattern was observed for the carbohydrate-rich seromucoid fraction. The results suggest that unidentified carbohydrate-rich proteins of the globulin fraction are either degraded more rapidly and synthesized more slowly than carbohydrate-poor globulins or are preferentially utilized for tissue maintenance and growth under conditions of acute protein demand.

Depletion by Tumor Growth and Caloric Restriction due to Anorexia

Our interest in the influence of dietary protein on the protein-bound carbohydrates and proteins of serum originated in observations made on rats bearing the highly malignant Walker 256 carcinosarcoma. In animals with far advanced tumors nonseromucoid glycoprotein/protein ratios were significantly

decreased.²⁴ This finding suggested that there might be preferential utilization of certain carbohydrate-containing proteins for tumor growth or their degradation to meet the increased energy demands of the tumor-bearing host.²⁵ This hypothesis has received support from the tissue culture studies of Kent and Gey,²⁶ who observed a selective uptake of globulin glycoprotein by two strains of rat tumor cells, one of which was the Walker 256 carcinosarcoma.

The results of a serial study are presented below. Animals were inoculated subcutaneously at the midline of the back with a suspension of the tumor. They were maintained on Purina laboratory chow and tap water ad lib. Groups of rats were sacrificed at intervals. A synopsis of the data obtained on the animals is given in TABLE 12.

Marked decreases had occurred in hemoglobin and hematocrit levels by the sixth day following transplantation. At this time the tumor weight constituted 1 per cent of the total body weight.

TABLE 12
GENERAL AND HEMATOLOGIC DATA

Group	N-0	T-3	T-6	T-10	T-14	T-20	T-22	N-22
No. rats	15	14	14	15	15	14	15	15
Tumor growth (days)	0	3	6	10	14	20	22	0
Hematocrit (%)	47 ± 0.5	45 ± 0.9	43 ± 0.6*	31 ± 1.0*	29 ± 0.9*	33 ± 1.9*	33 ± 1.2*	46 ± 0.5
Hemoglobin (gm. %)	15.1 ± 0.17	15.2 ± 0.21	13.9 ± 0.23*	9.4 ± 0.37*	8.1 ± 0.22*	7.7 ± 0.43*	7.9 ± 0.30*	14.5 ± 0.14*

Findings include the standard error of the mean. Significant differences from normal values (N-0) are indicated as follows:

* $P = < 0.1$.

FIGURE 4 presents the carcass and tumor weight curves. The carcass continued to grow at a normal rate for 2 weeks following tumor inoculation. At this time anorexia developed and there was a decrease in food consumption from 23 gm. per day at day 15 to 4 gm. per day at day 22.

FIGURES 3a, b, and c summarize the results of total protein determinations and the electrophoretic analyses. The first significant change was the transient increase in the β -globulin fraction in the T-3 group. Albumin and α_1 -globulin levels were significantly decreased 6 and 10 days following tumor transplantation respectively. Of interest was the unanticipated return of the albumin fraction to normal levels in the latter stages of tumor growth. This was reflected in the biphasic curve for total protein and the increased A/G ratio. The minor electrophoretic fractions, α_2 and γ globulins were not affected by tumor growth. The experiment was terminated at day 22 following the spontaneous death of a number of animals on the preceding day.

FIGURE 5 shows the results of protein-bound carbohydrate analyses. Total serum glycoprotein (TSG) concentrations were significantly reduced 10 days postinoculation. They then returned to normal as a result of increased levels of seromucoid hexose (SMH). However, if SMH values are subtracted from

TSG concentrations, the resultant curve, TSG-SMH, remains at T-10 levels. FIGURE 6 summarizes the corresponding calculations for total serum protein (TSP) and seromucoid protein (SMP). In this case the resulting curve, TSP-

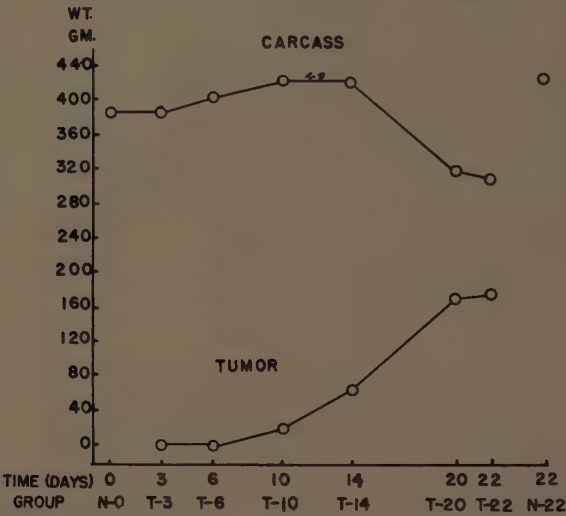


FIGURE 4. Residual carcass and tumor weight curves.

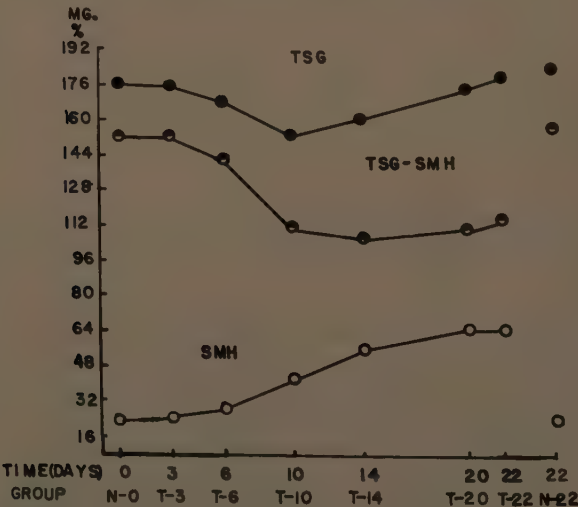


FIGURE 5. Effects of tumor growth on the protein-bound carbohydrates of serum. Key: ●, total serum glycoprotein; ○, seromucoid hexose; ○, total serum glycoprotein minus seromucoid hexose.

SMP, does not diverge in inflection points from the TSP curve. TABLE 13 presents the results of glycoprotein/protein ratio computations. There was a significant increase in the untreated ratio, TSG/TSP, during tumor growth. When seromucoid values were subtracted, significant decreases occurred in the ratio as tumor growth progressed. The above data corroborate Winzler's²⁷

concept that different glycoproteins may have divergent physiologic or pathologic roles. It is suggested that the increased seromucoid levels may reflect a systemic response to tissue injury and/or cellular proliferation. The decreased nonseromucoid glycoprotein/protein ratios are due in part to the increase in the carbohydrate-poor albumin fraction (FIGURE 3b). However, it is apparent that the decreased ratio also reflects the preferential utilization of unidentified

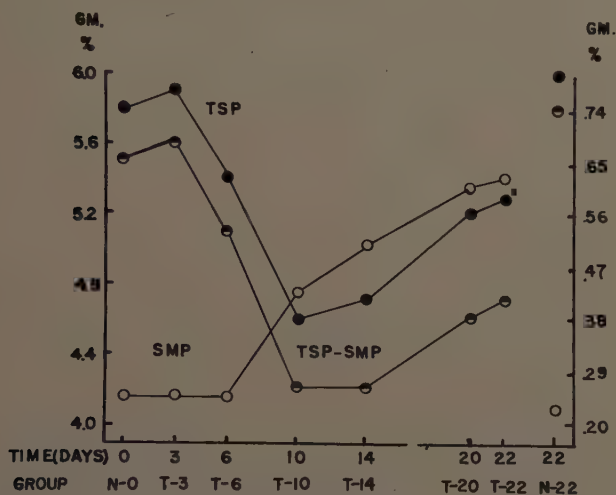


FIGURE 6. Effects of tumor growth on serum proteins. Key: ●, total serum protein; ○, seromucoid protein; ◐, total serum protein minus seromucoid protein.

TABLE 13
SERUM GLYCOPROTEIN/PROTEIN RATIOS IN TUMOR-BEARING RATS

Group	N-0	T-3	T-6	T-10	T-14	T-20	T-22	N-22
TSG/TSP × 100 (%)	3.0 ± 0.05	3.0 ± 0.05	3.1 ± 0.04	3.3 ± 0.08*	3.4 ± 0.11*	3.3 ± 0.05*	3.4 ± 0.09*	3.0 ± 0.05
TSG-SMH/TSP-SMP × 100 (%)	2.7 ± 0.05	2.7 ± 0.05	2.8 ± 0.04	2.6 ± 0.06	2.5 ± 0.09*	2.4 ± 0.04*	2.4 ± 0.05*	2.7 ± 0.05

Findings include the standard error of the mean. Significant difference from normal values are indicated as follows:

* $P = <0.01$.

carbohydrate-rich protein(s) of the globulin fraction to meet the demands for tumor growth and carcass maintenance in the depleted host.

Serum Glycoprotein Response in Tumor-Bearing Rats on a Protein-Free Diet

A previous study demonstrated (FIGURE 5) that in rats bearing a highly malignant tumor, the serum glycoprotein response is characterized by significant increases in the seromucoid fraction and marked decreases in the non-seromucoid glycoproteins. Protein depletion caused significant decreases in the protein-bound carbohydrates of serum. Restriction of dietary protein has

also been found to reduce the rate of growth of experimental tumors.²⁸ The effect of protein depletion on the serum glycoprotein response of the host to tumor growth has apparently never been elucidated. The following study was initiated to determine the effects of protein depletion on the serum protein-bound carbohydrates and proteins of rats bearing the Walker 256 carcinosarcoma. Three categories of controls were included; normal rats fed a stock diet (Group A), tumor-bearing animals maintained on a stock diet (Groups B and C), normal rats fed a protein-free diet until their carcass weight loss approximated that of the experimental animals (Group D).

Two groups of rats were fed a protein-free diet until weight losses of 12 (Group E) and 23 per cent (Group F) were attained. They were then inoculated with a suspension of the tumor and continued on the diet. The animals were sacrificed after 14 days of tumor growth.

General and hematologic data are presented in TABLE 14. The degree of protein depletion at the time of tumor inoculation had little effect on the final weight of the tumor (cf. E and F). In both tumor-bearing groups fed the protein-free diet, however, the size of the tumor was significantly less than that of the control group (B) maintained on the stock diet. Hemoglobin and hematocrit levels were significantly reduced in all tumor-bearing rats irrespective of diet. Hemoglobin values were also somewhat lower than in the group (D) subjected to protein-depletion only.

Total serum glycoprotein concentrations were subnormal in all of the 14-day tumor-bearing groups (TABLE 15). While a significant decline in total serum protein levels occurred in all groups subjected to experimental procedures, the decreases were most pronounced in the tumor-bearing groups maintained on the protein-free diet.

The components of the seromucoid fraction increased significantly in all tumor-bearing groups irrespective of diet. However, nonseromucoid glycoprotein levels were significantly reduced in all experimental groups, and similar results were noted for nonseromucoid protein values. The decreases were most pronounced in Groups E and F.

Glycoprotein/protein ratios for total serum were significantly increased in all tumor-bearing groups. Divergent results were obtained for nonseromucoid glycoprotein/protein ratios. Two of the tumor-bearing groups exhibited significant decreases and two were in the normal range.

The results of the electrophoretic analyses are summarized in TABLE 16. Consistent findings were the significant decreases in the albumin and α_1 -globulin fractions of all experimental groups. Of interest was the observation that in Group F, the α_1 -globulin concentration exceeded that of albumin.

The results demonstrate that even though the rate of tumor growth is restricted by feeding a protein-free diet, the response of the seromucoid fraction is not affected. The combination of protein depletion and tumor growth caused a significantly greater reduction in nonseromucoid glycoprotein levels than was observed in tumor-bearing or in protein-depleted animals.

Effects of Protein Depletion on the Antibody Response in Normal and Tumor-Bearing Rats

The investigations of Cannon and his associates^{12,20} have emphasized the importance of a high level of dietary protein for the production of antibodies.

TABLE 14
GENERAL AND HEMATOLOGIC DATA

Group	No. rats	Initial wt. (gm.)	Inoculation wt. (gm.)	Duration tumor growth (days)	Final carcass wt. (gm.)	Tumor wt. (gm.)	Hemoglobin (gm. %)	Hematocrit (%)
Stock diet								
A. Normal	15				386 ± 5.0		15.1 ± 0.16	47 ± 0.5
B. Tumor-bearing	15		386 ± 4.5	14	413 ± 6.9	67 ± 4.7	8.1 ± 0.21*	29 ± 0.9*
C. Tumor-bearing	14		386 ± 5.4	20	320 ± 10.8*	167 ± 7.9	7.7 ± 0.43*	33 ± 1.9*
Protein-free diet								
D. Normal	9				266 ± 4.0*		11.4 ± 0.36*	36 ± 1.4*
E. Tumor-bearing	14		352 ± 4.3*	14	281 ± 6.3*	47 ± 5.2	10.0 ± 0.34*	35 ± 1.6*
F. Tumor-bearing	15		302 ± 3.4*	14	249 ± 3.4*	37 ± 2.1	9.0 ± 0.16*	30 ± 0.6*

Results include the standard error of the mean.

Significant differences from normal values (Group A) are indicated as follows:

* $P = < 0.01$.TABLE 15
SUMMARY OF CHEMICAL DATA

Group	Total serum			Serumucoid fraction			Nonserumucoid		
	Glycoprotein (mg. %)	Protein (gm. %)	G/P ratio (%)	Hexose (mg. %)	Protein (gm. %)	H/P ratio (%)	Glycoprotein (mg. %)	Protein (gm. %)	G/P ratio (%)
Stock diet									
A. Normal	175 ± 3.3	5.8 ± 0.06	3.0 ± 0.05	24 ± 0.5	0.25 ± 0.006	9.6 ± 0.20	151 ± 3.1	5.5 ± 0.06	2.7 ± 0.05
B. Tumor-bearing	161 ± 3.3†	4.7 ± 0.08†	3.4 ± 0.11†	54 ± 1.8†	0.51 ± 0.012†	10.6 ± 0.13†	107 ± 2.3†	4.2 ± 0.08†	2.6 ± 0.10
C. Tumor-bearing	173 ± 3.9	5.2 ± 0.16†	3.3 ± 0.05†	63 ± 1.9†	0.61 ± 0.022†	10.3 ± 0.33	110 ± 0.4†	4.6 ± 0.12†	2.4 ± 0.05†
Protein-free diet									
D. Normal	131 ± 2.5†	4.7 ± 0.06†	2.8 ± 0.04†	23 ± 1.4	0.26 ± 0.016	8.8 ± 0.19†	108 ± 1.8†	4.4 ± 0.06†	2.4 ± 0.04†
E. Tumor-bearing	144 ± 3.7†	4.2 ± 0.12†	3.4 ± 0.09†	55 ± 3.0†	0.55 ± 0.030†	10.0 ± 0.25	89 ± 3.9†	3.4 ± 0.13†	2.4 ± 0.06†
F. Tumor-bearing	160 ± 4.9†	4.1 ± 0.12†	3.9 ± 0.11†	76 ± 2.8†	0.63 ± 0.024†	12.1 ± 0.41†	88 ± 3.0†	3.4 ± 0.12†	2.6 ± 0.07

Findings include the standard error of the mean.

Significant differences from normal (Group A) values are indicated as follows:

† $P = < 0.01$.‡ $P = < 0.05 > 0.01$.

TABLE 16
RESULTS OF ELECTROPHORETIC ANALYSES

Group	Total serum protein (gm. %)	Albumin (gm. %)	Globulin				A/G ratio
			α_1 (gm. %)	α_2 (gm. %)	β (gm. %)	γ (gm. %)	
Stock diet							
A. Normal	5.8 \pm 0.06	2.4 \pm 0.08	1.8 \pm 0.07	0.5 \pm 0.06	0.9 \pm 0.05	0.2 \pm 0.06	0.71 \pm 0.08
B. Tumor-bearing	4.7 \pm 0.08†	1.8 \pm 0.08†	1.3 \pm 0.03†	0.6 \pm 0.03	0.8 \pm 0.04	0.2 \pm 0.02	0.62 \pm 0.04
C. Tumor-bearing Protein-free diet	5.2 \pm 0.16†	2.1 \pm 0.11‡	1.4 \pm 0.06†	0.6 \pm 0.05	0.9 \pm 0.06	0.2 \pm 0.02	0.68 \pm 0.04
D. Normal	4.7 \pm 0.06†	1.5 \pm 0.08†	1.0 \pm 0.04†	0.7 \pm 0.06	1.0 \pm 0.04	0.5 \pm 0.06†	0.47 \pm 0.03†
E. Tumor-bearing	4.2 \pm 0.12†	1.5 \pm 0.09†	1.0 \pm 0.05†	0.6 \pm 0.05	0.8 \pm 0.06	0.3 \pm 0.04	0.58 \pm 0.05†
F. Tumor-bearing	4.1 \pm 0.12†	1.0 \pm 0.07†	1.2 \pm 0.04†	0.8 \pm 0.07†	0.9 \pm 0.08	0.2 \pm 0.03	0.32 \pm 0.03†

Findings include the standard error of the mean.

Significant differences from normal values (Group A) are indicated as follows:

† $P = < 0.01$.

‡ $P = < 0.05 > 0.01$.

This concept has not gone unchallenged.⁶ In Cannon's studies,^{12,20} animals were fed a 2 per cent protein diet (chronic depletion), a nutritional regimen that requires considerable time to effect depletion. Quantitative differences in serum protein and glycoproteins between rats subjected to a protein-free diet (acute depletion) or chronic depletion have been reported.²¹ Antibody production has therefore been investigated in both normal and tumor-bearing animals maintained on a protein-free diet. Groups of rats were fed either the stock diet (A,B) or a protein-free diet (C,D,E). After a weight loss of 25 per cent in the depleted groups, immunization with a 1 per cent suspension of *Salmonella typhosa* H antigen was begun. All immunized animals were administered 2.5 ml. of the antigen in a series of 6 subcutaneous and intraperitoneal injections over a period of 2 weeks. One week after starting the antigen injections, Group E was inoculated with a suspension of the Walker 256 car-

TABLE 17
SUMMARY OF GENERAL AND HEMATOLOGIC DATA

Group	No. rats	Initial wt. (gm.)	25 per cent depletion wt. (gm.)	Final wt. (gm.)	Hemoglobin (gm. %)	Hematocrit (%)
Stock diet						
A. Control (saline-injected)	16	371 ± 2.9		412 ± 3.6	14.6 ± 0.60	47 ± 0.3
B. Immunized Protein-free diet	22	373 ± 1.9		401 ± 3.3	14.1 ± 0.15	47 ± 0.4
C. Control	9	401 ± 6.6	303 ± 4.2	266 ± 3.8	11.4 ± 0.36	36 ± 1.4
D. Immunized	14	402 ± 5.8	303 ± 4.2	268 ± 3.6	11.9 ± 0.17	37 ± 1.1
E. Immunized (tumor-bearing)	19	407 ± 4.2	304 ± 3.7	259* ± 3.3	8.9 ± 0.13*	29 ± 0.6†

* Carcass Weight. Tumor weight = 34.8 ± 1.9 gm. Findings include the standard error of the mean.

Significant differences between Control Group C and Groups D and E are indicated as follows:

† $P = < 0.01$.

cinosarcoma. The animals were bled by cardiac puncture one week following the final antigen injection.

General and hematologic data are presented in TABLE 17. No significant differences in weight and in hematologic values were found between the immunized groups and their corresponding controls. In the group (E) subjected to the additional stress of tumor-bearing, hematologic values were conspicuously low. A summary of the immunologic and chemical data is shown in TABLE 18. Feeding a protein-free diet (D) caused a 64 per cent decrease in the level of circulating antibodies. The agglutinin titer was further depressed in the animals that were also tumor-bearing (E). Both groups, however, demonstrated an appreciable response to antigen injection. It may be inferred that the antibody response is substantially reduced only under very drastic circumstances. The degree of resistance to challenge with viable *S. typhosa* was not investigated.

The rats employed in these experiments, although obtained from our customary source, had serum values somewhat higher than those reported in the preceding experiments (TABLE 18). Glycoprotein/protein ratios for the normal

TABLE 18
SUMMARY OF IMMUNOLOGIC AND CHEMICAL DATA

Group	Agglutinin titer		Total serum			Serumucoid fraction	
	Mean	Range	Glycoprotein (mg. %)	Protein (gm. %)	G/P ratio (%)	Hexose (mg. %)	Protein (gm. %)
Stock diet A. Control (saline-injected) B. Immunized	1:3900	1:1280-1:10,240	186 ± 3.4	6.6 ± 0.05	2.8 ± 0.06	ND	ND
			184 ± 2.4	6.6 ± 0.05	2.8 ± 0.05		
Protein-free diet C. Control D. Immunized E. Immunized (tumor-bearing)	1:1400 1:500	1:640-1:2560 1:80-1:1280	131 ± 2.5	4.7 ± 0.06	2.8 ± 0.04	23 ± 1.4	0.26 ± 0.016
			139 ± 3.1	4.6 ± 0.10	3.0 ± 0.07	30 ± 1.9*	0.33 ± 0.020*
			152 ± 2.6*	3.6 ± 0.08*	4.2 ± 0.17*	70 ± 1.4*	0.70 ± 0.017*

Chemical data include the standard error of the mean.
Significant differences between Control Group C and Groups D and E are indicated as follows:
* $P = <0.01$.

group, however, were in the normal range. The anticipated effects of protein depletion and tumor growth on serum protein and glycoprotein concentrations were observed. The immunized animals fed the stock diet (B) exhibited no divergences from normal in total serum protein and glycoprotein values. Similar observations have been made following the immunization of rabbits.²⁹ Alterations in the subfractions of serum as demonstrated by electrophoretic analyses did occur as a result of antigen injection (TABLE 19). Seromucoid values for the immunized group on the protein-free diet (D) were significantly greater than its corresponding control group (C). A shortage of serum precluded these analyses for Groups A and B. A significant increase occurred in γ globulin in the immunized rats concomitant with a significant decrease in the α_1 -globulin fraction. The decline in serum albumin levels following immunization which has been reported for many species²⁹ did not occur.

TABLE 19
EFFECTS OF IMMUNIZATION ON THE SERUM ELECTROPHORETIC PATTERN
OF THE ADULT RAT

Group	No. rats	Total serum protein (gm. %)	Albumin (gm. %)	Globulin				A/G ratio
				α_1 (gm. %)	α_2 (gm. %)	β (gm. %)	γ (gm. %)	
A. Control (saline-injected)	16	6.6 \pm 0.05	2.4 \pm 0.02	2.2 \pm 0.01	0.5 \pm 0.02	1.1 \pm 0.06	0.4 \pm 0.01	0.57 \pm 0.011
B. Immunized	17	6.6 \pm 0.05	2.4 \pm 0.02	1.9 \pm 0.01*	0.6 \pm 0.03	1.1 \pm 0.04	0.6 \pm 0.03*	0.57 \pm 0.014

Results include the standard error of the mean. Significant differences from control group are indicated as follows:

* $P = <0.01$.

Summary

The studies reported above have been concerned with the influence of dietary protein on the concentration and distribution of the serum proteins and glycoproteins of the adult, male Sprague-Dawley rat. Facets investigated included the effects of (1) the degree of protein depletion and repletion, (2) the level of dietary protein and carbohydrate, (3) tumor growth, and (4) antibody production.

Protein depletion is characterized by general decreases in the serum proteins and glycoproteins. Albumin and α_1 -globulin, the major electrophoretic fractions of the Sprague-Dawley rat are most sensitive to nutritional influence. The nutritional state and previous nutritional history of the animal affect the concentration and distribution of the carbohydrate-containing proteins of serum.

The protein-bound carbohydrates of serum are decreased to a greater extent than the protein components in moderate (25 per cent) depletion. Upon repletion, serum protein concentrations are restored more rapidly than the serum glycoproteins. The protein-bound carbohydrates of the globulin fraction continue to increase in the postrepletion period. It may be inferred from the data that the marked increases in the α - and β -globulin fractions and in the

serum glycoproteins observed in many pathologic states are not the result of reduced protein intake. The level of dietary carbohydrate does not influence serum glycoprotein levels, indicating that these conjugated proteins do not play a role in carbohydrate transport.

In rats depleted by tumor growth the albumin and α_1 -globulin fractions are decreased during the early stages. With the advent of anorexia, a premortal rise occurred in serum albumin. The components of the seromucoid fraction are significantly increased in tumor-bearing rats and this response is not affected by feeding a protein-free diet. This finding suggests that there is no failure of synthesis of protein-bound carbohydrates during protein depletion.

Antibody production is impaired but not abolished in protein-depleted and tumor-bearing animals. The decreased nonseromucoid glycoprotein/protein and globulin polysaccharide/protein ratios in rats during protein depletion and repletion, and in animals bearing large tumors, is consistent with the concept that some unidentified carbohydrate-rich protein(s) in the globulin fraction may be preferentially utilized for the growth and maintenance of both somatic and tumor cells.

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THE EFFECT OF DIET ON THE MATURATION OF THE NEONATAL PIGLET'S SERUM PROTEIN PROFILE AND RESISTANCE TO DISEASE*

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The work described in this paper grew from difficulties encountered in raising colostrum-free pigs for subsequent use in experiments dealing with either infectious disease¹ or mineral metabolism.² To secure such pigs, they either were removed from the sow approximately 2 days before term by hysterectomy or were caught in sterile towels at parturition. Immediately following birth, colostrum-free pigs were carried to another isolation unit and placed individually in sterilized stainless steel cages where they were fed 6 times a day on a diet consisting of cow's milk supplemented with minerals and vitamins. In spite of what was considered strict, sanitary conditions, it was not unusual to lose 30 to 60 per cent of the litter. Generally, diarrhea was observed at about the third day of life, most of the pigs dying between the fourth and the eighth day. *Escherichia coli*, type 08, frequently were isolated from the blood of morbid and the liver of dead pigs, as well as from the feces of healthy, vigorous pigs.³ Little future trouble was encountered in raising piglets that survived the first 8 days.

It was possible to circumvent the mortality of the first week, either by continuously treating the pigs with intramuscular injections of chloromycetin or by limiting the intake of food, coupled with intraperitoneal injections of porcine gamma globulin.⁴ Although these "tricks" minimized death losses, the lack of weight gain and poor appearance of survivors impressed on us the need for questioning the adequacy of cow's milk as a food for baby pigs. Nursing pigs born devoid of gamma globulin obtain from the sow a colostrum rich in gamma globulin, whereas pigs fed cow's milk are deprived of this important bulwark against disease. The importance of colostrum might be inferred from the work with calves, for it has been shown that cow's colostrum provides the calf, also born essentially gamma globulin-free, with immune globulin. It is thought that the immune globulin contains antibodies to *E. coli* and thereby protects the calf from diarrhea and death.^{5,6}

Without minimizing the role and the importance of antibodies in disease resistance, it was our intent to investigate other possibilities whereby the mammary secretions of the sow might contribute to the disease resistance of the piglet. Two observations stimulated this approach: (1) even though it was possible to keep piglets alive during the critical first week, the colostrum-free piglets had gained little or no weight, while piglets nursing the sow had doubled their weight in this interval; and (2) coincident with approximately a week of age colostrum-free piglets started growing and little future trouble was encountered in raising them. Nevertheless, they were still antibody-free pigs and

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were to remain antibody-free for a few more weeks.⁷ Obviously, antibody was not all important to the viability of the piglet. It was our concept, then, that the sow was contributing specialized nutrients to the piglet in addition to antibody, and these nutrients were not found in a "balanced" diet such as cow's milk. Furthermore, a lack of these specialized nutrients (as reflected in lower weight gains) poised the susceptibility of the piglet to bacterial invasion from the gut. Thus our experiments were designed to determine whether the diarrhea, bacteremia, and death of piglets was preceded by an interruption in normal physiological development resulting from a deficient diet.

To circumscribe an experimental approach, a measurement of dietary effect more specific than weight gain seemed desirable. A comparison of the piglet's neonatal serum protein profile with that of an adult pig's indicated an avenue exploitable for this purpose. Serum profiles were characterized by three measurements: (1) concentration of albumin, alpha, beta, and gamma globulin; (2) per cent total protein; and (3) optical density (O.D.) of trichloroacetic acid (TCA) precipitate. In the neonatal pig (FIGURE 1), the serum proteins were described by a profile that was low in total protein, in albumin and beta globulin, contained no gamma globulin, and was high in alpha globulin. The majority of protein present at the time of birth was not precipitated by TCA.⁴ Incidentally, the neonatal profile is much more stark than the neonatal profile of other animals born essentially gamma globulin-free, for example, the calf, the goat, and the sheep.^{8,9,10} These animals are born with approximately 6 times more albumin than the pig.

An early experiment was designed to determine the time required and the changes involved in going from the "immature" serum protein profile of birth to the "mature" profile of an adult.⁴ We also wished to know the effect of various diets on the development of a mature profile. To accomplish this, serum protein profiles were noted at 0, 2, 4, 7, and 14 days of age in pigs kept on 4 different diets. One group of pigs was allowed to nurse the sow under normal conditions, a second group was fed cow's milk, a third group was fed an "artificial" milk (made up to simulate cow's milk) containing cow's casein, lactalbumin, lactose, fat, minerals, and vitamins, and a fourth group was fed an "amino acid" milk similar in composition to the artificial milk except that the milk proteins were enzymatically hydrolyzed to their constituent amino acids and short-chained peptides. The amino acid milk was formulated anticipating the piglet's problem to be one of an inability to digest proteins in the first few days of life. As may be seen in a summary of these results (FIGURE 2), pigs nursing the sow had immediate and continuing changes in serum proteins rapidly leading (within 14 days) to the formation of a mature serum profile. Conversely, piglets fed cow's milk experienced a latency of from 4 to 7 days in the maturation of the serum protein profile. Pigs fed the artificial milk had an even longer delay, and those fed the amino acid diet were totally unable to affect their immature serum protein profile.

Those pigs (nurslings) with the rapidly maturing serum protein profile gained weight rapidly and were least affected by disease, while those pigs with the static, immature profile gained little or no weight and, unless medicated during this latent period, they were most susceptible to diarrhea, bacteremia, and death. Coincident with the start of serum protein development (approximate

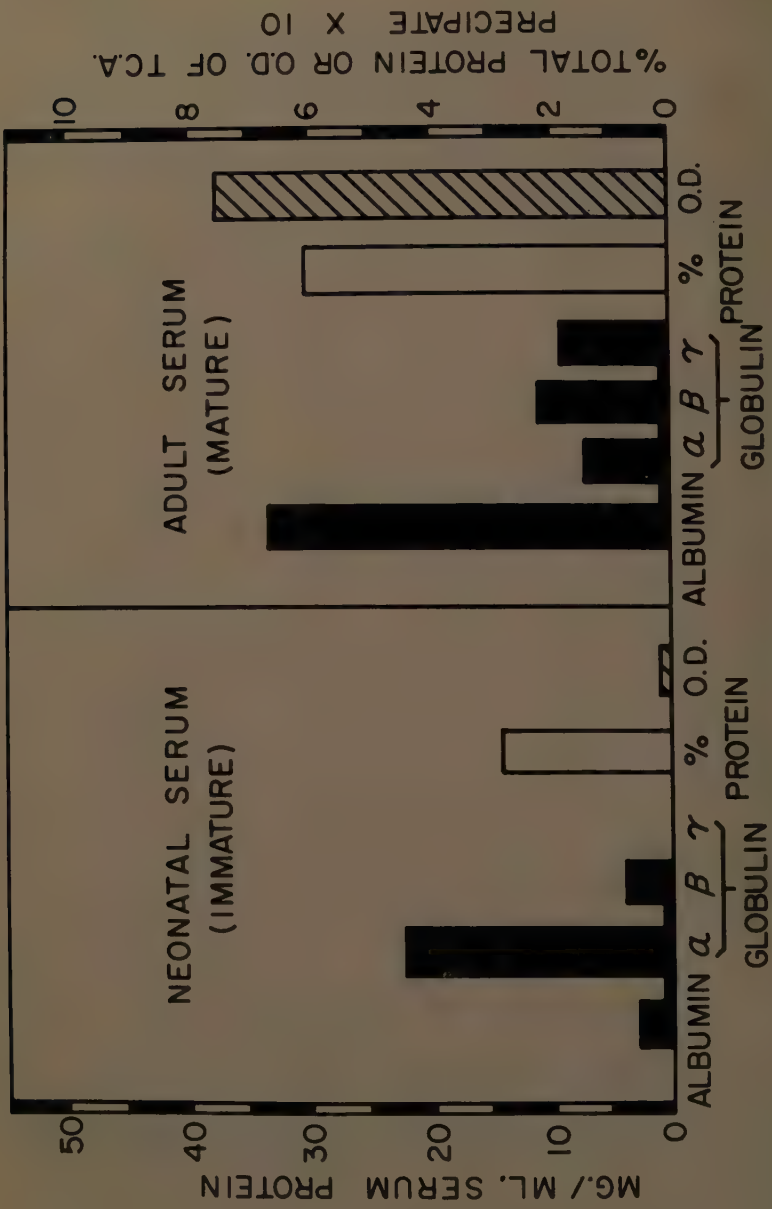


FIGURE 1. Porcine neonatal and adult serum protein profiles.

mately one week of age in piglets fed cow's milk), medication was no longer necessary and weight increased in these retarded piglets.

The neonatal piglet's serum protein profile, coupled with the changes involved in the maturation of the serum protein profile, were reminiscent of the serum protein profile found in fetal sheep and goats at the end of the first third of their gestation and the changes these serum protein profiles made while the fetus was maturing.^{9,10,11} However, the maturation of the postbirth piglet's serum pro-

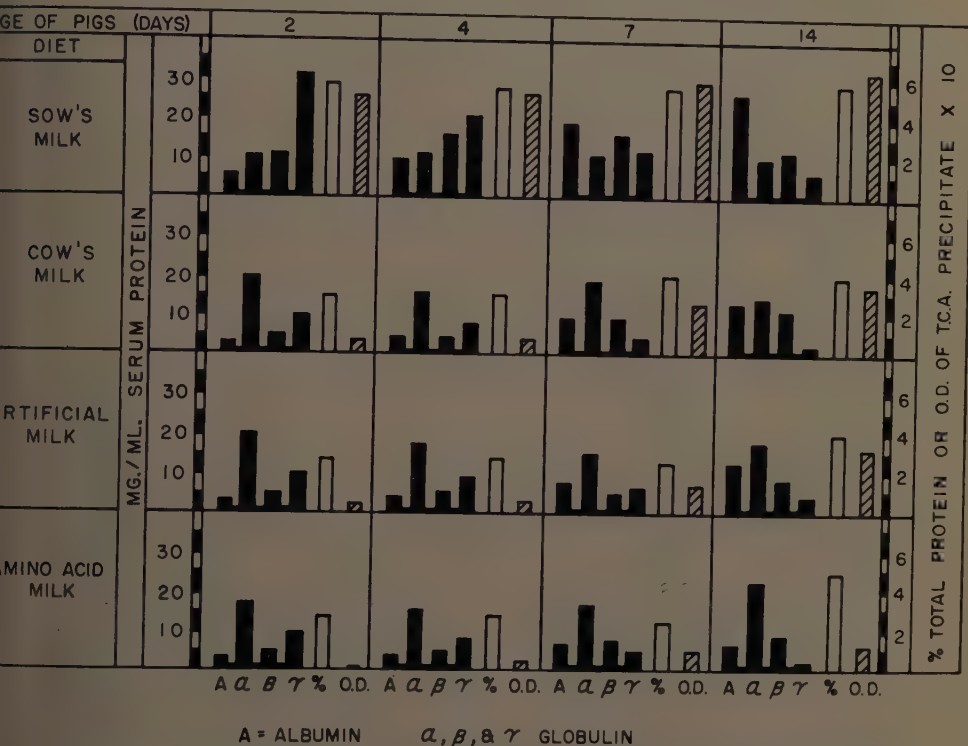


FIGURE 2. The effect of diet on the maturation of the piglet's serum protein profile. Pigs, with exception of nurslings, were injected with 15 ml. of 10% porcine gamma globulin at birth.

tein profile occurred at a faster rate than the more leisurely developing profile in developing sheep and goat fetuses.

Obviously the sow, through her mammary secretions, is markedly influencing the maturation of the piglet's serum protein profile. The desire to mimic these changes in piglets, independent of the sow, led us to the next phase of our work: namely, learning by what means the sow influences the piglet's serum protein profile. Contrast of the whey proteins found in sow's colostrum with the neonatal serum protein profile (FIGURE 3) showed that one was practically the reciprocal image of the other: where neonatal serum was low in protein fractions, the colostrum was high and vice versa. The relationship of these patterns, coupled with the rapid changes in serum proteins of nursing piglets, suggested

that the initial changes in the piglet's pattern were directly influenced by the kinds of proteins present in the colostrum; furthermore, that the rapid changes resulted from the absorption of whole colostrum proteins from the gut of the piglet. This view was supported by data demonstrating the absorption of antibody in neonates of many species.¹²

To confirm the view that unaltered proteins were absorbed from the piglet's gut and to describe further the means whereby the sow influences piglet's serum protein profiles, we desired some insight regarding the specificity of the early absorption phenomenon. For example, for absorption to take place, must the

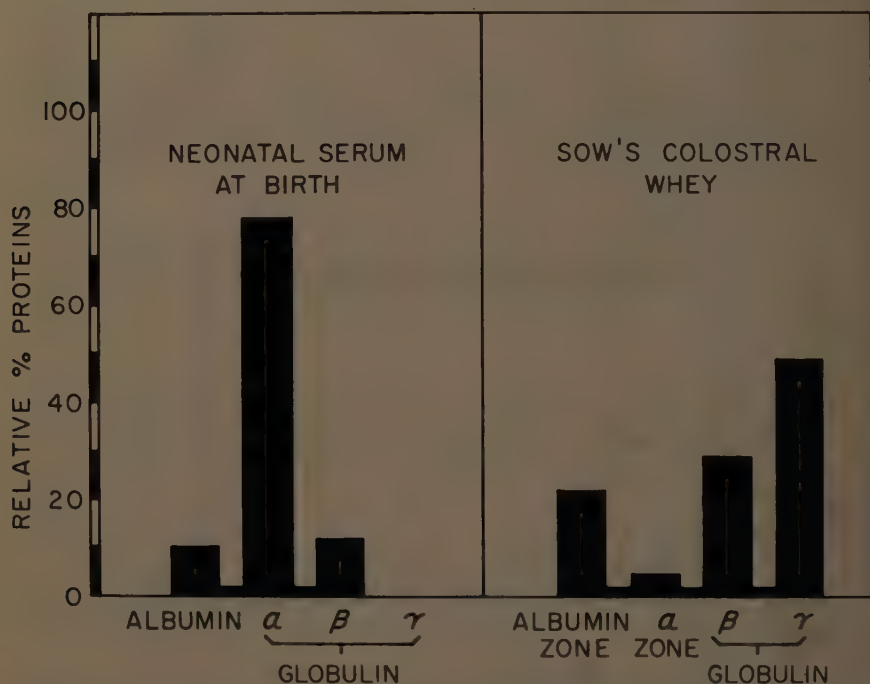


FIGURE 3. Relative concentration of porcine neonatal serum proteins and colostral whey proteins.

protein be homologous with respect to function and species, or is the absorption phase analogous to a screen with holes large enough to allow the transport of any large protein molecule independent of source and function, or—for that matter—need the large molecule be protein? To test these possibilities, pigs were fed from the time of birth three different kinds of high molecular weight materials.¹³ The first was bovine colostrum. This was fed because it contains protein functionally similar but from a different species mainly, bovine gamma globulin. Next, whole avian eggs were fed because the species is far removed phylogenetically from pigs; and the protein, particularly ovalbumin, probably is different functionally from sow's colostral proteins. The last large molecular material fed was polyvinylpyrrolidone (P.V.P.), a synthetic, high molecular weight, blood plasma expander.

Immunoelectrophoresis was used to detect the absorption of egg proteins (FIGURE 4) and bovine colostral proteins (FIGURE 5), while agar electrophoresis was used for the detection of P.V.P. (FIGURE 6). A summary of the results indicated that the early absorption mechanism in the piglet, qualitatively at least, is completely nonselective. Major and minor bovine colostral proteins and egg proteins, both of the albumin and globulin type, were subsequently demonstrable in the serum of neonatal pigs eating these proteins, as was also

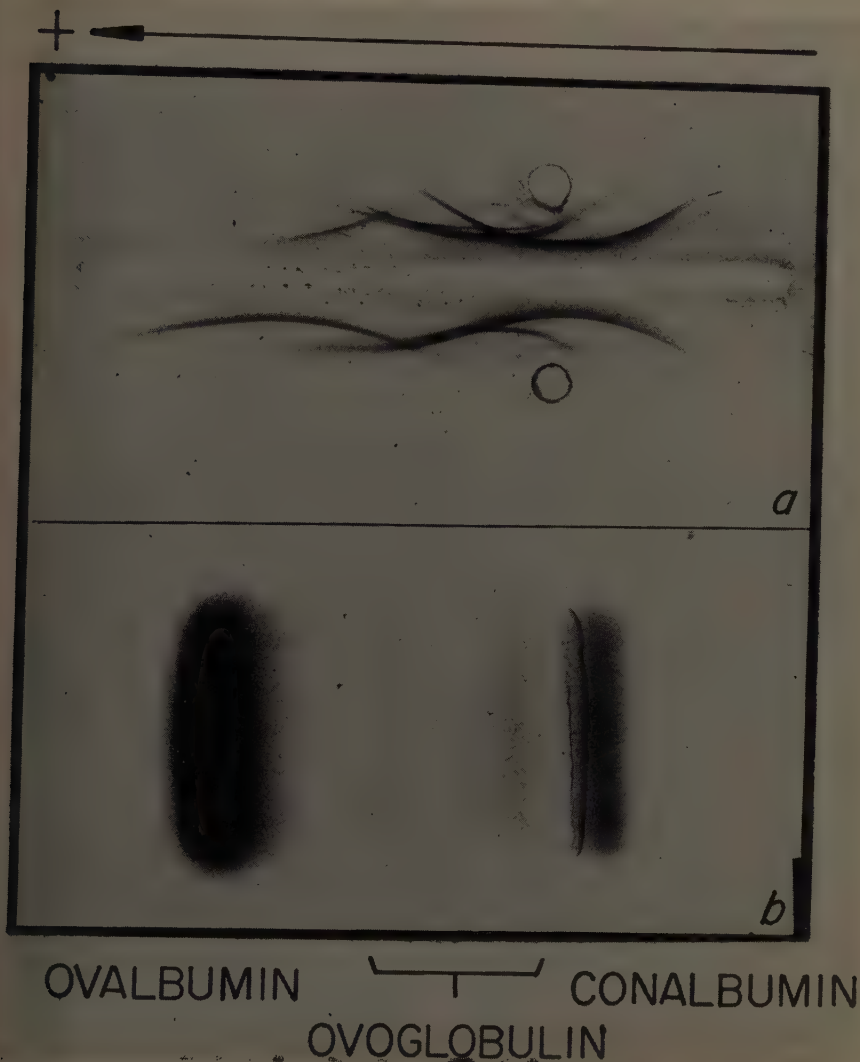
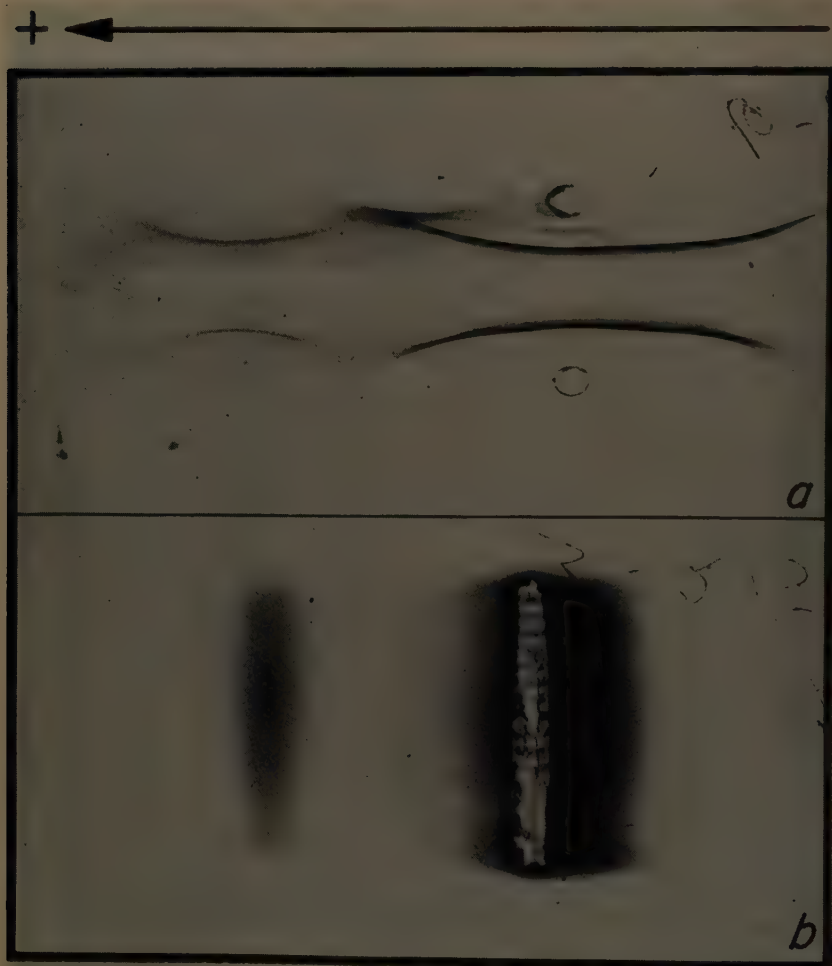


FIGURE 4. (a) Immunoelectrophoresis for the detection of egg proteins in piglet's serum. Top hole, egg white; bottom hole, serum from pig fed egg; center trough, anti-egg-white serum. (b) Agar electrophoresis of egg white proteins. Arrow indicates direction of electrophoretic mobility at pH 8.6, Veronal buffer, ionic strength 0.05.

the high molecular weight, blood plasma expander, P.V.P. This nonselectivity is not peculiar to piglets since calves fed whole egg behave in a similar manner (J. G. Lecce and D. O. Morgan, unpublished data). No doubt then, the absorption mechanism existing in the piglet, coupled with the proteins found in sow's colostrum, accounts for the rapid serum protein changes occurring within the first 36 hours of the piglet's life. In effect, colostral proteins were in the piglet's gut one minute and, the next minute, in the piglet's serum.



LACTALBUMIN IMMUNE GLOBULIN

FIGURE 5. (a) Immunoelectrophoresis for the detection of bovine colostral proteins in piglet's serum. Top hole, bovine colostrum; bottom hole, serum from pig fed bovine colostrum; center trough, antibovine whey serum. (b) Agar electrophoresis of bovine colostral whey. Arrow indicates direction of electrophoretic mobility at pH 8.6, Veronal buffer, ionic strength 0.05.

Beyond this initial absorption phase, however, we wondered whether the sow still continued to influence the maturation of the piglet's serum profile. The following experiment was designed to test this.¹⁴ Pigs were weaned at 1, 4, 8, and 14 days to the cow's milk diet and the amino acid diet. This was done to determine whether the initial changes obtained from the absorption of whole protein from the sow's colostrum before the piglet was 36 hours old were all that were necessary to continue the uninterrupted maturation of the serum protein profile. The results indicated that little or no advantage in the maturation of

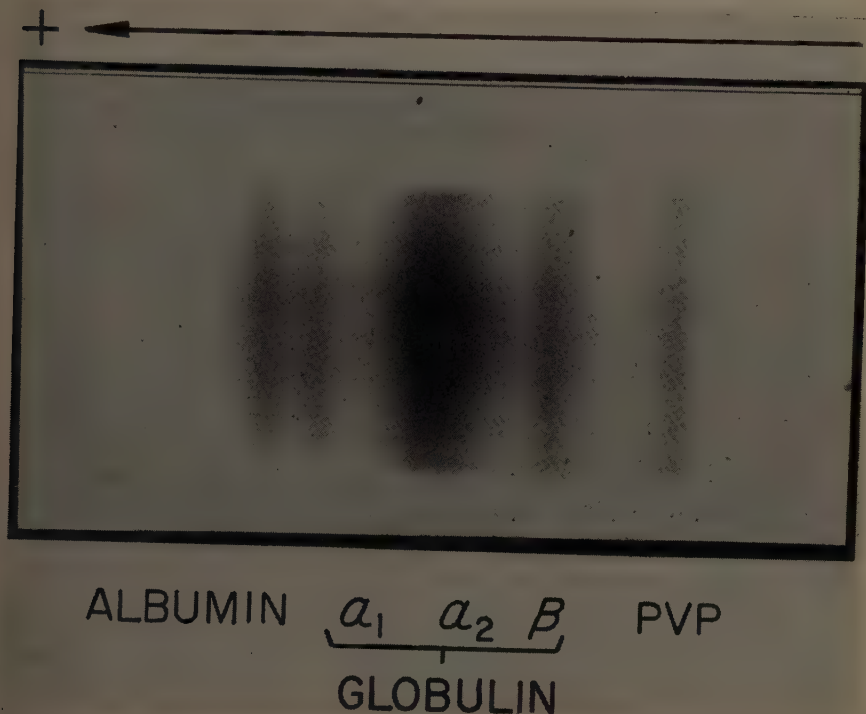


FIGURE 6. Agar electrophoresis of serum from 2-day-old piglet fed P.V.P. Arrow indicates direction of electrophoretic mobility at pH 8.6, Veronal buffer, ionic strength 0.05.

the serum protein profile accrued to piglets nursing for 24 hours, although ample initial changes were demonstrable. In these piglets, the initial changes regressed, and a latency in the development of the serum profile similar to the latency in piglets weaned directly to cow's milk was seen. Also, at this stage of physiological maturity, the "amino acid diet" (as was true in piglets weaned to this diet at birth) did not sustain the serum protein profile nor did it maintain life. Pigs nursing the sow for 4 days, however, seemed to have been sufficiently mature to sustain and continue the development of an adult serum protein profile on either diet. Note (FIGURE 7), a lag in the formation of serum albumin in pigs weaned at 1 day in comparison with pigs weaned at 4 days.

It was concluded from these data that the sow influences the maturation of

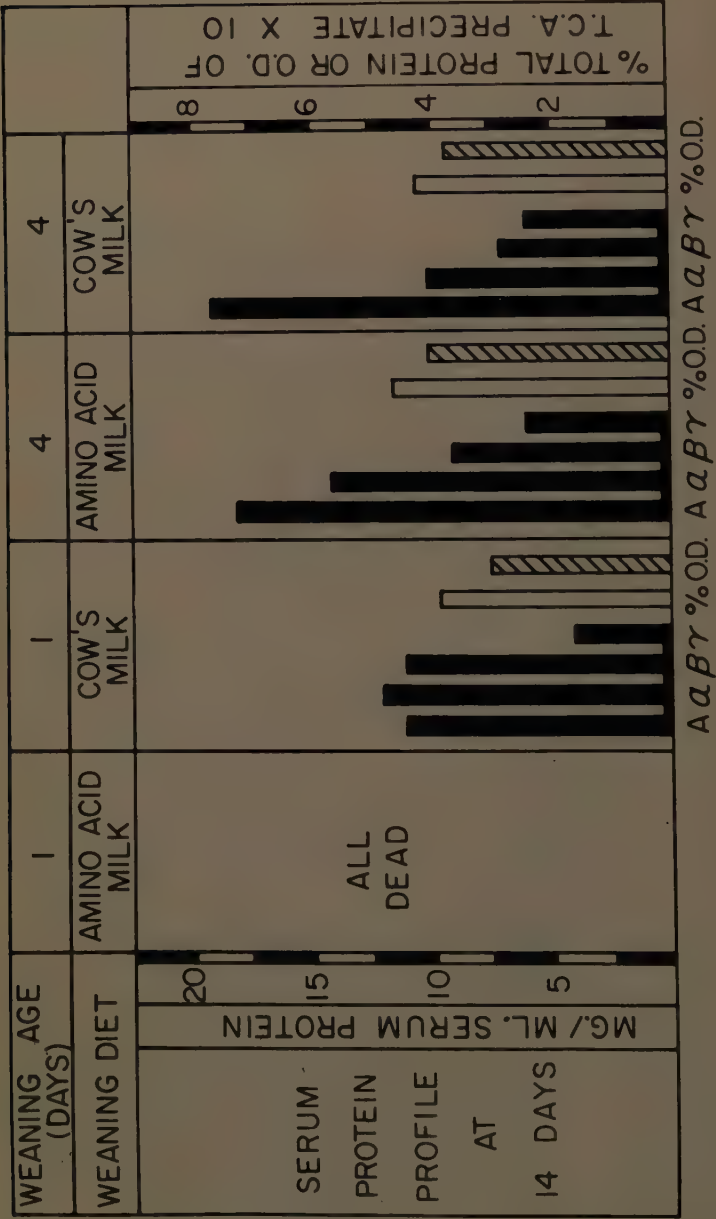


FIGURE 7. The effect on the serum protein profile at 14 days of weaning piglets at 1 and 4 days to an amino-acid diet and a cow's-milk diet.

piglet's serum protein profile in 2 different phases. The first phase results from the absorption of unaltered colostral proteins and this phenomenon is finished essentially after the first 36 hours. The second phase seems likely to be involved with a "balance of nutrients" or peptide moieties particularly rich in sow's milk.

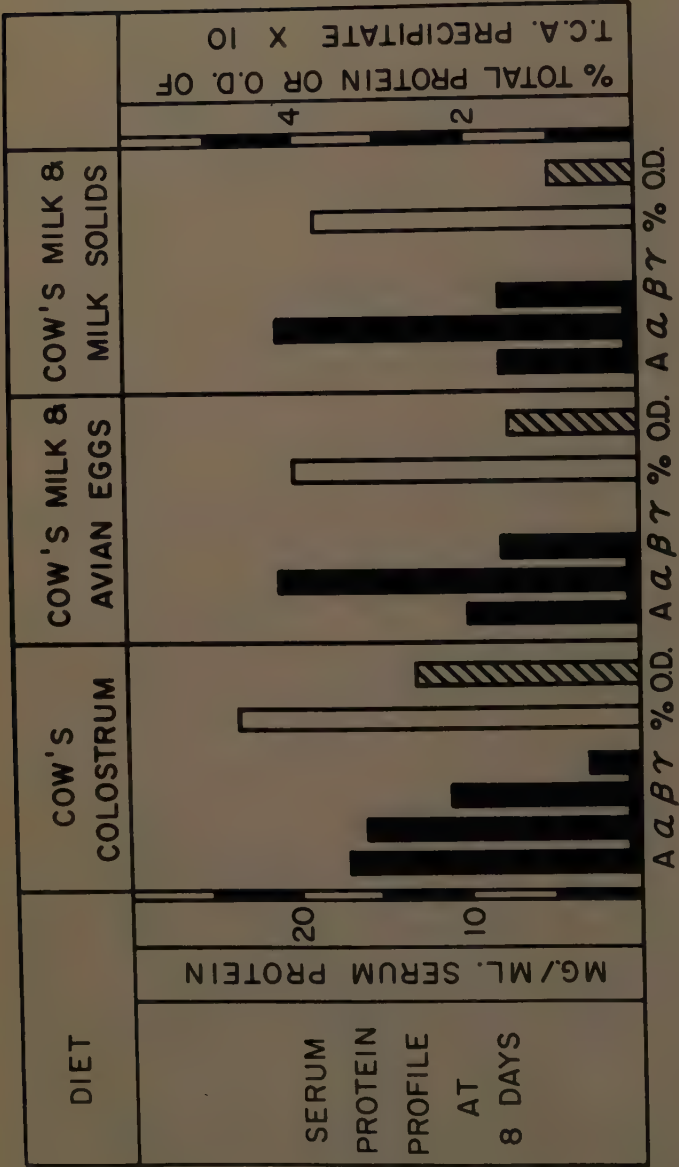
At this point, we felt we had enough information to permit a rational approach to mimicking the serum protein changes seen in pigs nursing the sow.¹³ This was done by feeding neonatal pigs two different diets: (1) cow's colostrum, and (2) cow's milk fortified with avian eggs. Increasing amounts of cow's milk was added to these diets so that, by 7 days, the piglets in all groups were being fed 100 per cent cow's milk. This is approximately the length of time required for the sow to change gradually from colostral to milk secretions. A third or control diet consisted of cow's milk fortified with whole milk solids so that it would roughly compare to the total protein solids present in the other two diets.

The results showed that pigs on the cow's colostral regime had initial and continuous changes in their serum protein profiles similar to pigs nursing the sow. Pigs nursing the sow, however, sustained these changes at a higher level. Although pigs fed egg had initial serum protein changes resulting from the absorption of egg proteins, there was no indication that these serum proteins had any favorable influence on the serum protein maturation process. In this group there was a latency of serum protein development similar to that occurring in the control pigs that were fed milk. FIGURE 8 illustrates the difference at 8 days in the maturation of the serum protein profile in pigs kept on these 3 diets.

In terms of performance, those pigs (cow's colostrum-fed) able to continue the maturation process after initial absorption of proteins gained the most and none died. On the other hand, pigs fed egg and milk demonstrated arrested serum protein development, gained less, and 60 and 57 per cent respectively died within weeks. These data expressed as weight-gain viability clearly illustrated this difference in performance (FIGURE 9).

In considering the problem of protein profile maturation further, we wondered why some of the fractions making up the neonatal, immature, serum protein profile were low. Two broad explanations occurred to us: (1) the electrophoretic fraction was low because many of the subfractions making it up were low, or (2) the electrophoretic fraction was low because many of the subfractions making it up were missing. Obviously, an analyzing technique that easily separated serum proteins into a maximum number of subfractions was needed to follow this line of questioning. We accordingly turned to immunoelectrophoresis. In our system we were able to detect, within the four main electrophoretic fractions of albumin, alpha, beta, and gamma globulin, 21 distinct subfractions.

A comparison of the neonatal, immature pattern with the adult, mature pattern indicated, within the limit of sensitivity of our technique, that the neonatal profile is not only quantitatively different from a mature pattern but it is qualitatively different as well (FIGURE 10). Quantitatively, the neonatal pattern (as reported earlier) is low in albumin, beta, and gamma globulin and high in alpha globulin. Qualitatively, there were approximately 12 less sub-



α, β, γ GLOBULIN

A = ALBUMIN

FIGURE 8. The effect on the serum protein profile at 8 days of feeding piglets cow's colostrum, cow's milk fortified with avian eggs, and cow's milk fortified with skimmed milk solids.

fractions in the neonatal serum: 1 is missing in the albumin, 4 in the alpha zone, 5 in beta, and 2 in the gamma.

These results led to the question that was really germane to the problem: namely (using immunoelectrophoretic fractions as criteria) can the changes involved in going from the immature to the mature pattern be influenced by

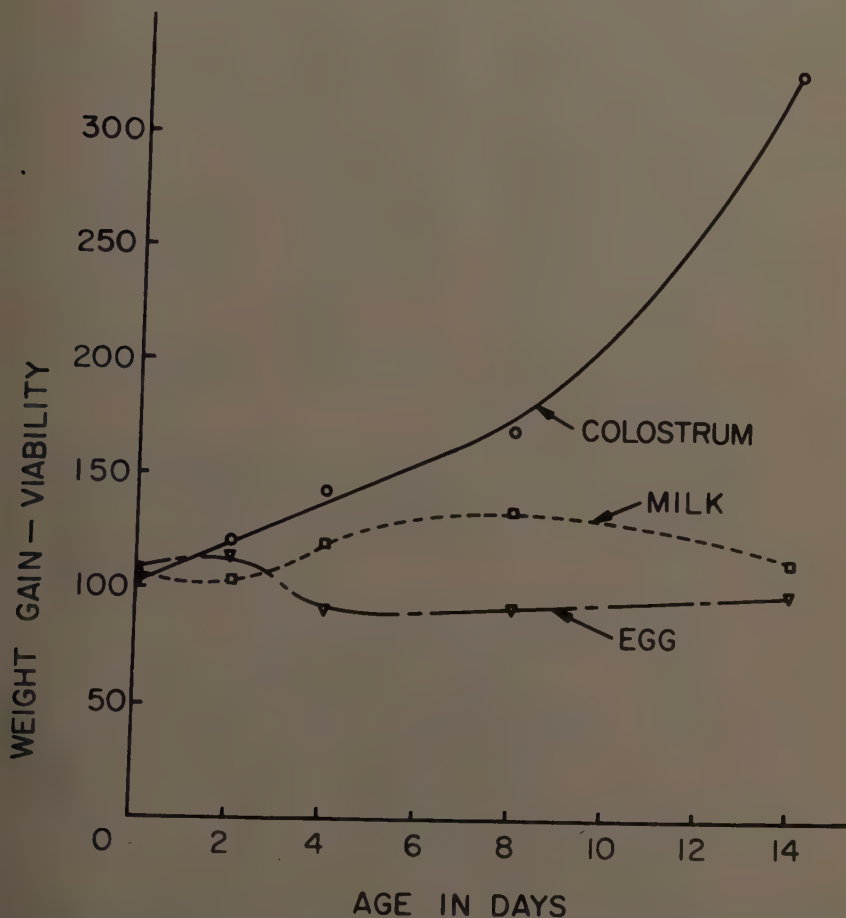


FIGURE 9. Weight-gain viability (mean weight \times per cent survival) of pigs fed cow's colostrum, cow's milk fortified with avian eggs, and cow's milk fortified with skimmed milk solids.

diet, that is, does the sequential appearance of subfractions occur independent of diet? If diet cannot influence the appearance of subfractions, this would indicate that the piglet with the latent serum protein profile had the ability to synthesize the same kinds of proteins seen in rapidly maturing piglets but just not in sufficient amounts to effect the quantity of protein in the profile. The problem would then seem one of supplying "building blocks" in amounts sufficient for optimal synthesis of serum proteins rather than a problem of enzymatic insufficiency.

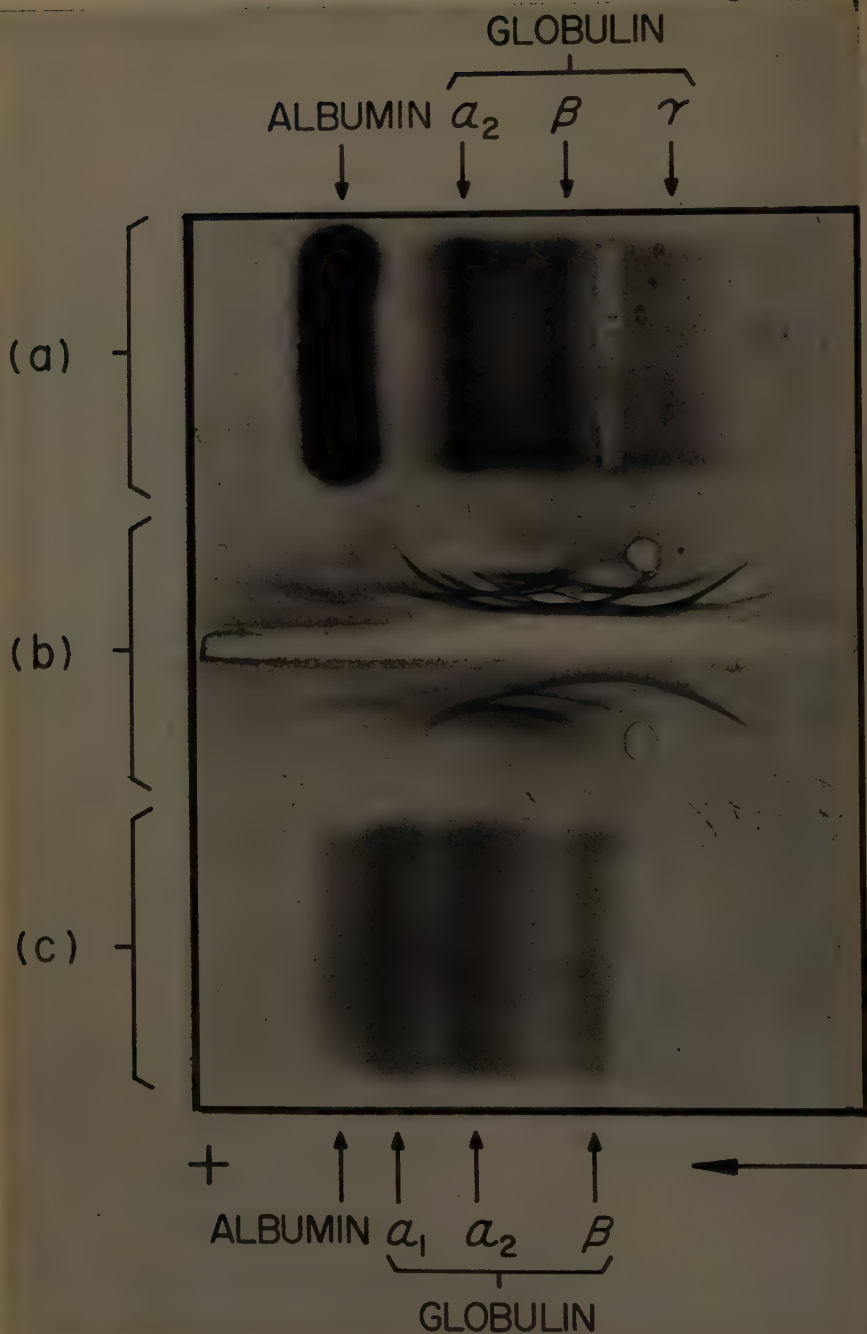


FIGURE 10. Agar and immunoelectrophoresis of porcine neonatal and adult serum. Arrow indicates direction of electrophoretic mobility at pH 8.6, Veronal buffer, ionic strength 0.05. (a) Agar electrophoresis of adult serum. (b) Immunoelectrophoresis of adult and neonatal serum. Top hole, adult serum; bottom hole, neonatal serum; center trough, anti-adult-porcine serum. (c) Agar electrophoresis of neonatal serum.

To answer the questions posed above, a set of standards were prepared from serum obtained from what we considered ideal, healthy, fast-growing pigs with rapidly maturing serum protein profiles. These were, of course, pigs that were nursing the sow. The serum obtained from these pigs at zero, 2, 4, 8, 22 days of age (and sometimes older) were compared with serum with a similar time sequence obtained from piglets on a variety of diets, in various states of health, and latency with regard to serum protein profile maturation. In essence, regardless of whether the piglets were widely different from the nursing pigs with respect to health and vigor and relative amounts of electrophoretic serum protein fractions, they were remarkably constant and similar to the ideal, rapidly maturing group in terms of the appearance of subfractions. These data were interpreted to indicate, for reasons just stated, that the piglet with the arrested serum protein profile had the capacity to synthesize the many different serum proteins at about the same time as the pigs with rapidly maturing serum protein profiles, but that he lacked serum protein quantity because of limited "building blocks."

In conclusion, work reported herein indicates that rapidly maturing serum protein profiles are to be found in healthy, vigorous, fast-growing piglets and arrested immature profiles in morbid piglets. Whether these serum proteins (with the exception of gamma globulin) play a direct role in disease resistance or whether the changes merely indicate the general nutritional state of an animal is not yet discernible. In essence, our problem with the piglet is one of an interaction between nutrition and infectious disease: the complexity of which, after three years of work, has not failed to impress us. And the overriding theme woven throughout our experiments is the detection of physiologic imbalance that shifts the resistance of the host to that of susceptibility to invasion by microorganisms.

Acknowledgment

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DIETARY PROTEIN AND ITS EFFECTS ON THE SERUM PROTEINS OF THE CHICKEN

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Introduction

Studies concerning the influence of dietary factors on serum proteins must by their nature be considered in light of the concepts of protein reserves and a dynamic state of protein metabolism. The studies of Allison¹ and Whipple² have demonstrated the importance of dietary proteins in maintaining the protein stores of the body. These studies in the dog and rat have shown that the serum-protein level and, more specifically, the level of serum albumin generally serve as a criterion of the protein reserve status of the animal.

The previously mentioned studies^{1,2} demonstrate that in the rat and dog the serum-protein level varies directly with the level of dietary protein. The variations found in serum protein as a result of altering the dietary nitrogen intake can be related almost exclusively to changes in the albumin level with a relatively constant level of globulins. This is particularly evident when changes in plasma volume are taken into account.³

Although considerable data have accumulated for the dog and rat relating changes in serum proteins to dietary protein, there have been few reports on this subject for the chicken.

Experimental Studies

The influence of dietary fat, cholesterol, and protein on the serum proteins of the growing chick has been investigated.⁴ Fat and cholesterol were found to be without effect on the serum-protein level; however, dietary protein significantly altered the serum-protein level.

A semipurified diet was employed in which soybean oil meal, properly supplemented with methionine, served as the protein source; two protein levels, 10 and 25 per cent, were fed. Other dietary variables included, at each protein level, changes in the level and type of fat and the presence or absence of cholesterol. Since fat and cholesterol exerted no influence on serum proteins, the body weight and serum-protein values have been calculated with dietary protein as the only variable; these data are presented in TABLE 1. It is evident from these values that increasing the dietary protein level from 10 to 25 per cent resulted in an increase in body weight and an increased serum protein and albumin level; the globulin fraction, however, remained essentially unchanged.

A second study was initiated to examine further the effect of the dietary-protein level on serum proteins in the chick.⁵ The diet differed from that used in the previous study in that sesame meal, adequately supplemented with lysine, served as the protein source. Four levels of protein were fed: 10, 15, 20, and 25 per cent of the diet. FIGURE 1 demonstrates the influence of these

protein levels on the body weight of the chick. The excellent fit of the regression line, calculated for the 3 lower protein levels, is evident. The lack of difference in body weight between the chicks fed the 20 and 25 per cent protein-containing diets indicates that the protein requirement for maximum growth

TABLE 1
INFLUENCE OF DIETARY PROTEIN ON BODY WEIGHT AND SERUM PROTEIN
IN THE CHICK

Dietary protein (%)	7-Week body wt. (gm.)	Serum protein*	Albumin*	Globulins*		A/G
				Alpha	Beta + gamma	
10	462	2.23	0.90	0.23	0.84	0.84
25	1154	3.20	1.92	0.20	0.90	1.74

* Gm./100 ml.

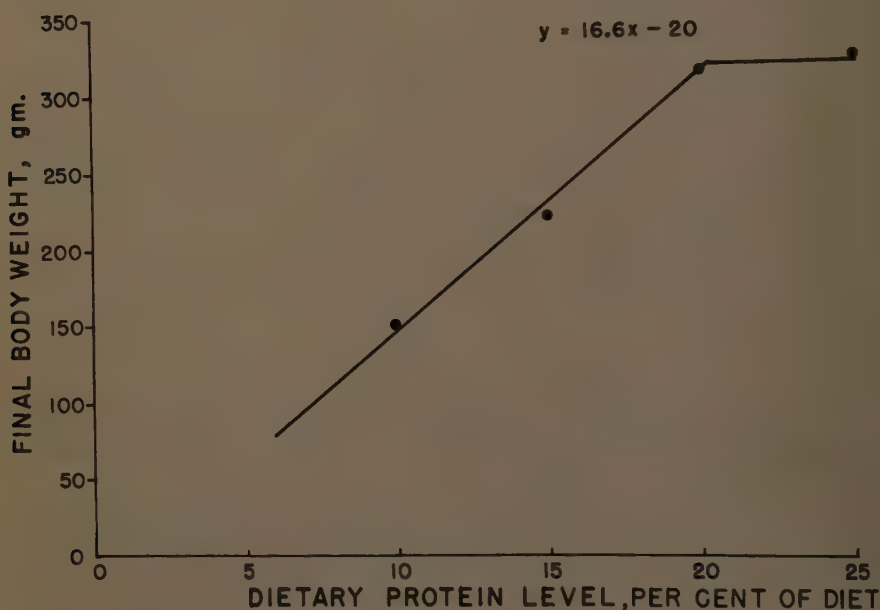


FIGURE 1. Growth of chicks fed differing levels of protein.

was no greater than 20 per cent (the requirement calculated from FIGURE 1 was 20.5 per cent).

The data presented in FIGURE 2 demonstrate the influence of the dietary protein levels employed on serum protein. There was a linear increase in total serum protein for all dietary levels employed. The serum proteins increased from 2.33 gm. per cent at the 10 per cent protein level to 3.06 gm. per cent at the 25 per cent level of protein. The data on the electrophoretic components are presented in TABLE 2, together with body weight, serum protein, and plasma

volume data. These data are in accord with those of the previous study in that the only electrophoretic component to vary was albumin. The increasing level of albumin accompanying the higher levels of dietary protein and the stable globulin levels resulted in increasing A/G (albumin to globulin) ratios.

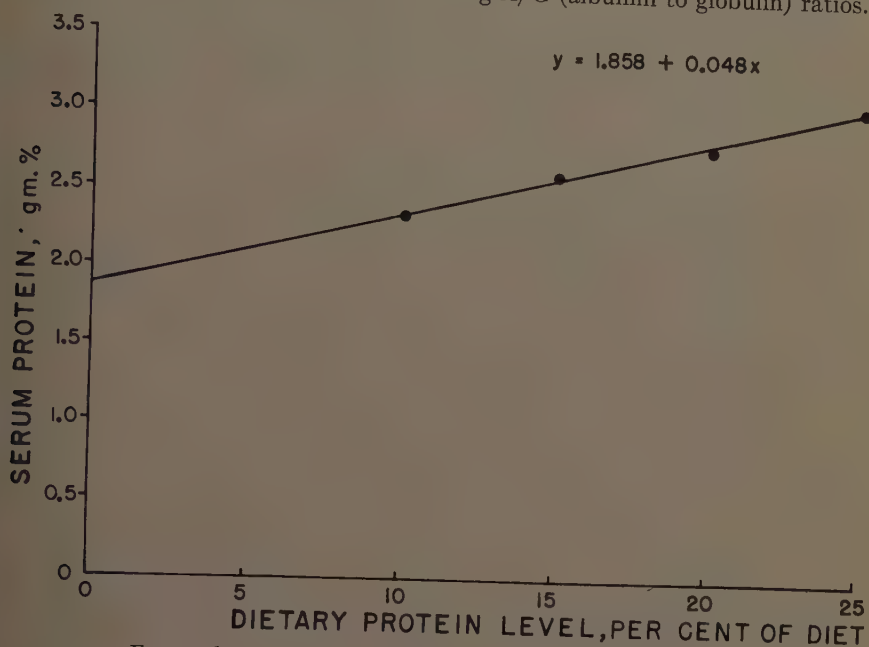


FIGURE 2. Influence of dietary protein on serum-protein level.

TABLE 2

INFLUENCE OF DIETARY PROTEIN ON SERUM-PROTEIN COMPONENTS, BODY WEIGHT, AND PLASMA VOLUME OF THE GROWING CHICK

Dietary protein level (%)	4-Week body wt. (gm.)	Total serum protein*	Albumin*	Globulins*		A/G	Plasma volume (% of body wt.)
				Alpha	Beta + gamma		
10	156 ± 33†	2.33 ± 0.32	1.22 ± 0.24	0.52 ± 0.11	0.55 ± 0.22	1.14	—
15	221 ± 40	2.60 ± 0.49	1.50 ± 0.38	0.50 ± 0.08	0.61 ± 0.21	1.35	7.2
20	316 ± 27	2.80 ± 0.11	1.78 ± 0.10	0.46 ± 0.05	0.56 ± 0.05	1.74	6.8
25	323 ± 21	3.06 ± 0.21	1.97 ± 0.19	0.50 ± 0.09	0.59 ± 0.06	1.81	7.1

* Gm./100 ml.

† Mean ± standard deviation.

It is of particular interest to note the increase in serum protein and albumin in chicks receiving 25 per cent protein, as compared to the 20 per cent protein level. This observation demonstrates that levels of protein above that required for maximum growth can support further increases in protein reserves. This indicates that the dietary protein requirement for maximum filling of the protein stores is in excess of that required for maximum growth.

It is also of significance to note (TABLE 2) that in the growing chick a level of protein less than that required for maximum growth does not result in a decreased plasma volume; this is in contrast to the protein-depleted rat.³

It is concluded from the above studies that total serum protein or albumin level, or A/G ratio, is a relatively accurate measure of the protein reserve status of the growing chick; also that the variations in total serum protein, resulting from changes in the dietary level of protein, are mediated primarily through alterations of the serum albumin.

Significance of Plasma Volume Changes

The influence of a change in plasma volume on the level of serum components expressed as units per volume of serum or plasma is evident. A decrease in plasma volume will result in an apparent increase of the various serum components.

That a decrease occurs in plasma volume during protein depletion has been demonstrated.³ Since the inanition accompanying various pathological states

TABLE 3
INFLUENCE OF NITROGEN SOURCE AND LEVEL ON SERUM-PROTEIN FRACTIONS
OF LAYING HENS

Diet	Plasma protein*	Albumin*	Globulins*			A/G
			Alpha	Beta + gamma	PP†	
Amino acid (1.5 % N)	4.2	0.49	0.39	1.64	.34	0.21
Practical (2.4 % N)	4.4	0.79	0.53	1.56	.34	0.32

* Gm./100 ml.

† Phospho-protein.

results in a depletion of the protein reserves, the possible influence of changes in plasma volume must be considered under these conditions. The effect of a reduced plasma volume is to show a lesser decrease in albumin than has actually occurred and increased globulin levels; the A/G ratio, however, would remain unchanged as has been pointed out by Allison.¹

Effect of Nitrogen Intake on Serum Proteins of Adult Hens

The influence of either a practical diet or an amino acid diet containing 2.5 or 1.4 per cent nitrogen, respectively, was studied in the laying hen.⁶ A decrease in the serum-albumin level was observed and, although variations were noted in certain globulin fractions (TABLE 3), the A/G ratio decreased markedly in hens fed the free amino acid diet, while the serum-protein level showed little change. These observations, particularly the altered A/G ratio, are indicative of a depletion of the protein stores and probably, because of the lack of change in total serum protein, of a loss of plasma water. Such a change in plasma volume would be in contrast to the observations noted above for the chick where the ingestion of a low-protein diet did not alter the plasma volume (as a percentage of body weight) significantly.

Various reports have appeared for the chick relating changes in serum protein to vitamin E deficiency^{7,8} and certain pathological conditions.⁹ However, all the data presented in these reports show decreased A/G ratios indicating a certain degree of depletion of the protein stores. The absence of a decrease in total serum protein (albumin decreases slightly, globulin fractions increase) indicates a loss of plasma water. All these observations strengthen the above statement concerning the significance of considering plasma volume changes when measuring serum components.

Significance of Protein Reserves

The data presented have demonstrated that an elevation in the level of dietary protein will result in increased protein reserves as judged by changes in total serum protein and, particularly, serum albumin and A/G ratio. Conversely, feeding low-nitrogen diets to laying hens and various deficiency and disease states appear to result in decreased protein stores, as indicated by decreased albumin levels and A/G ratios.

Such changes in protein reserves are of themselves of little consequence; they gain significance if it can be demonstrated that high protein reserves are of importance in meeting the stresses of disease, injury, and other similar conditions.

Data are available indicating that the protein reserves actually do serve in meeting such stress situations. The reports of Rosenthal and Allison¹⁰ and Rosenthal¹¹ demonstrate quite conclusively that the protein stores serve as an energy store in the sense that they enable the animals (dogs in these studies) to meet the stress of caloric restriction. The significance of this function of the protein reserves becomes apparent when one considers the number of disease conditions that are generally accompanied by some degree of caloric restriction due to decreased intakes.

The reports of Cannon *et al.*,^{12,13} demonstrating the inability of the protein-depleted rat or rabbit to produce antibodies at the same rate as the nondepleted animal, furnish another example of the value of protein reserves in meeting the stress of infection. That the protein stores serve a similar role in the chick has been demonstrated by Seeler and Ott.¹⁴ These authors presented data demonstrating that chicks fed low levels of protein were not able to survive infection with avian malaria as well as the controls receiving an adequate dietary protein level. The data of Cannon¹³ also indicate that the protein reserves are of value in meeting the stress of injury, in that the recovery rate from various surgical procedures in the human is well correlated to the preoperative nutritional status.

The evidence cited for the existence of reserve protein is subject to some criticism. The caloric value of the extra body protein reported^{10,11} does not denote any protein advantage to the animal. Similarly the work of Seeler and Ott¹⁴ might be interpreted to indicate a nonspecific effect of generally good nutrition on resistance to the pathogens under investigation. This can be inferred from data comparing the state of vitamin nutrition to resistance to disease. Finally, Halac¹⁵ has recently questioned the validity of the protein reserve concept by pointing out that survival of rats under protein-free feeding was not improved by previous high- versus low-protein feeding.

In view of these arguments, it is pertinent to cite the following direct evidence on protein reserves as obtained with the laying hen by Fisher *et al.*¹⁶ When hens were fed on diets containing either suboptimal protein or a suboptimal level of methionine, food consumption decreased sharply as soon as these diets were offered. Within one week egg production ceased but, concomitantly, food intake returned to normal levels. After a period of continuous normal food intake, egg production again commenced to be accompanied once more by a sharp decrease in voluntary food intake. TABLE 4 shows the details of such observations.

It is apparent that the inadequate diets led to food refusal, which in turn caused the cessation of egg production. With the stress of production removed, the diets were fully adequate to meet the maintenance needs, and the hens reacted by again consuming their full daily allotment. Quite obviously, protein reserves were built up during the pause in egg production, ultimately resulting in the renewed onset of production. It is therefore concluded that

TABLE 4
INFLUENCE OF PROTEIN LEVEL AND METHIONINE SUPPLEMENTATION ON EGG
PRODUCTION AND FEED CONSUMPTION IN THE LAYING HEN*

Week	Dietary variables			
	Protein level (%)		Methionine supplementation	
	12	15	None	±
First	3.6 (11)†	4.8 (3)	3.2 (6)	5.8 (1)
Second	1.0 (0)	2.8 (0)	0.8 (2)	4.6 (1)
Third	3.6 (2)	4.2 (0)	2.5 (3)	4.8 (2)
Fourth	3.4 (4)	3.4 (0)	—	4.6 (0)

* Average weekly egg production for 5 hens (eggs/hen).

† Numbers in parentheses represent the number of hen days on which sufficient feed had accumulated, due to refusals, obviating the need for the daily 100-gm. feed allotment.

protein reserves play a vital role in an animal such as the hen, which has such a large protein turnover in the form of eggs.

Summary

Data have been presented demonstrating that in the chicken the level of serum protein, serum albumin, and the A/G ratio all bear a direct relationship to the level of protein intake. It has been shown that the variations in serum protein are almost entirely due to changes in the albumin level, the globulins being rather stable and showing little or no variation. It has been pointed out that such changes in serum protein and serum-protein fractions are a reflection of the level of protein reserves.

The importance of plasma volume changes in interpreting serum-protein changes has been discussed.

The value of high protein reserves in meeting the stresses of caloric restriction, disease, infection, and injury has been discussed. Data have been presented demonstrating a role for protein stores in the hen with its rapid turnover of protein in egg formation.

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RECENT STUDIES ON THE SERUM PROTEINS OF GERMFREE ANIMALS*

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The germfree animal has been reared in the absence of any demonstrable microbial form. Thus it seems logical to expect that those serum protein fractions that normally harbor antimicrobial antibodies will show a certain deficiency or possibly be absent in the germfree animal. In 1958 I reported¹ that the absence of a microbial flora caused a deficit in α_2 , β , and γ globulin in the serum of the rat. In the chicken only a lower γ fraction was found, while the then available germfree guinea pigs showed no γ globulin whatsoever. These studies have now been extended to include other electrophoretic techniques, while more data are available on guinea pigs, rabbits and, also, ruminants.

Analysis of Germfree and Conventional Rat Serum

In conventional rat serum, as many as 13 different protein entities have been recognized thus far by immunoelectrophoretic techniques. These represent a diversity of origin and function. In the germfree rat, where the microbial stimulus is missing, there is the above-mentioned deficit in the globulin fractions. This deficit in serum globulins is compensated to a large extent by an increase, in absolute terms, of serum albumin.²

We assume that the presently available germfree rat is essentially a healthy animal.³ It grows well and has reproduced into the 15th generation. Its nutrition is considered adequate. The animal seems to be fairly free of undue environmental stress. The only obvious abnormality is an approximately fivefold increase in the size of the cecum, a condition apparently connected directly with the absence of an intestinal flora.⁴ While cholesterol metabolism is known to be affected by the presence or absence of a microbial flora,^{5,6} these changes do not seem to be such as to influence strongly the lipid-containing α - or β -globulin fractions. Some effect, especially in the α_2 fraction, could be expected, however. For the present purpose we shall regard the deficit in the various globulin fractions seen in the free-boundary electrophoresis pattern of germfree rat serum as directly related to the absence of a microflora and of the ensuing state of "physiological inflammation" found in the conventional animal. In conventional rats antimicrobial antibodies have been reported in both the β - and the γ -globulin fractions.^{7,8}

The classic free-boundary (F.B.) method separates rat serum into 5 or 6 fractions that can be quantitated in the time-honored way. The work by Grabar⁹ and others has shown however, that more, well-separated fractions

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exist, and that the Tiselius technique at best gives a rather crude summary of the actual situation. Immunoelectrophoresis (I.E.), on the other hand, gives a much more detailed picture of the serum proteins, but thus far adequate quantitation of the various discernible fractions has been lacking. It will be a means, however, to decide whether, in the absence of a microflora, the deficit seen in the F.B. pattern is caused by the virtual or absolute absence of one or more of the fractions discernible with the I.E. method.

In FIGURE 1 results of the F.B. method (Antweiler microelectrophoresis technique²) are compared with those of the I.E. technique, obtained with conventional rat serum. The I.E. pattern always shows at least the 10 lines indicated, with some variation in the α_2 -beta region. In most cases the

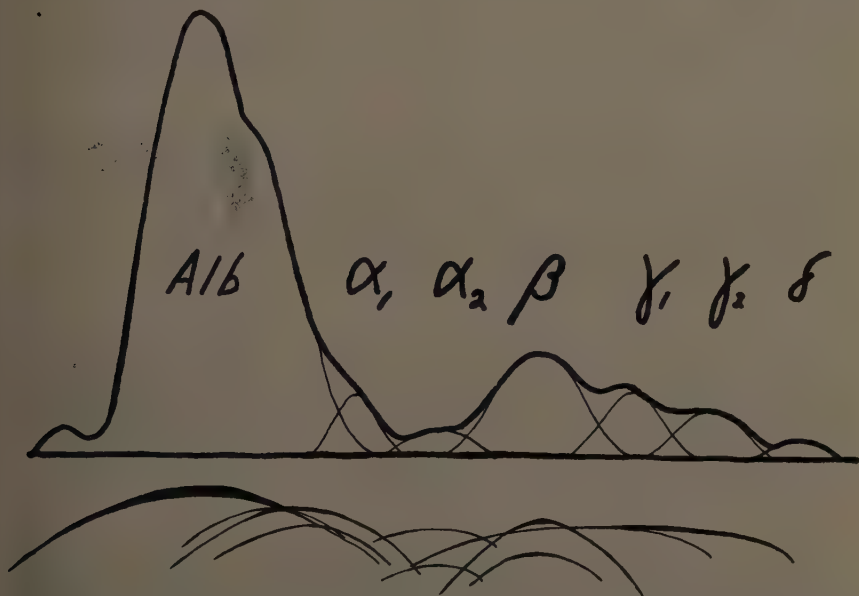


FIGURE 1. Comparison of free-boundary and immunoelectrophoretic patterns of conventional rat serum.

relationship with the F.B. fractions is clear. The patterns obtained with the Antweiler technique often, but not in all instances, show a subdivision of the albumin peak. In accordance with Moore *et al.*,¹⁰ this possible second peak was regarded as part of the albumin fraction. In the gamma range of the F.B. pattern in most instances two fractions are discernible that thus far have been evaluated as indicated in FIGURE 1. In the I.E. pattern of the rat serum, however, the gamma line, as in human serum, extends into the beta range and even touches the α_2 region. It becomes obvious that beta globulin values calculated in the usual way from the F.B. pattern are too high, while gamma-globulin values are too low. This apparently agrees with Gustafsson's data, which show 500 to 600 mg. per cent gamma globulin in conventional rat serum when determined with paper electrophoresis (a value that agrees well with our own data), but 700-800 mg. per cent when determined with immunological

methods.¹¹ However, although gamma-globulin values obtained with the F.B. technique may be too low, they seem to be representative for the gamma fraction and therefore useful. I disagree with Gustafsson when he suggests that the low beta-globulin values found in germfree rats may in essence be a lack of gamma globulin making itself felt in the beta range.¹² The fact that, upon exposure of the germfree rat to a "normal" flora, the beta-globulin frac-



FIGURE 2. Immunoelectrophoresis of germfree and conventional rat serum (see text). In wells: (a) germfree rat, (b) conventional rat, (c) conventional rat and (d) germfree rat. In troughs, anticonventional rat rabbit serum.

tion rises 1 to 2 weeks before the gamma fraction² shows that at least one "true" beta-globulin component must increase upon contact with the flora.

Although most of the time the gamma fraction presents itself in the I.E. pattern of the rat as a single line, very often we find an offshoot of this line in the slower-moving range (FIGURE 2c). This seems to indicate a superposition of lines indicating very similar but not identical fractions in the gamma range and could explain why two gamma fractions are rather consistently indicated in the F.B. patterns, although the present evaluation of these patterns must be

regarded as more or less incorrect (FIGURE 1). In some instances the I.E. pattern of the rat even shows a full separation into two gamma lines (FIGURE 2b). This phenomenon has also been reported to occur in the analysis of human serum.¹³ In the germfree rat the patterns in FIGURE 2 show in one case only a vague trace of a gamma fraction (FIGURE 2d) while, in another pattern developed at other concentration ranges, no line is visible in the gamma range (FIGURE 2a). Except for this difference in the gamma ranges, we have so far been unable to find any major differences between the patterns of the germfree and the conventional rat (compare FIGURES 2a and b). In the beta fraction all lines found in the I.E. of the serum of the conventional rat are also present in the germfree and even their position and intensity are not too different. Apparently all but the fraction indicated by the specific gamma lines are present in both sera and in amounts which in this method give comparable results. The question whether true gamma globulin is at all available in germfree serum was studied by Grabar at the Institut Pasteur, Paris, France. The serum of an animal that showed a questionable gamma response was used to produce antirat rabbit serum. Using a pure rat gamma-globulin preparation and this antiserum in the I.E. technique, a definite full-sized gamma precipitin line appears, apparently indicating that the germfree rat serum produced an entire spectrum of "antigamma" antibody in the rabbit (FIGURE 3).

The conclusion is reached that, even using the more refined immunoelectrophoresis method, the germfree rat appears to possess all serum protein fractions found in the conventional animal, even though the gamma fraction is strongly reduced. It would seem that this fraction contains most but certainly not all of the circulating antibody.

The F.B. pattern of the mouse strongly resembles that of the rat, although the alpha-globulin fraction is more prominent. The absence of the microflora results in, roughly, a one-third reduction of the beta globulin fraction and strong reduction of the gamma fraction. No change seems to take place in the alpha range.

Bovine Serum Albumin as an Antigen in the Germfree Rat

As mentioned before, exposure of the germfree rat to a complete microflora gives rise to consecutive increases in the alpha₂-, beta-, and gamma-globulin fractions seen in the F.B. electrophoresis pattern, in this sequence in time. Assuming that the increment in the various fractions is brought about by a direct reaction of the host to the presence of the microflora and its metabolites, this could indicate that various antibody producing mechanisms, reacting at different times to different stimuli, are involved. As has been indicated by Wagner, elsewhere in this monograph, monocontamination of germfree born rats at weaning with the bacterial species studied thus far (*Clostridium perfringens* types A and E, *Streptococcus faecalis* and *Lactobacillus casei*) did not visibly change the F.B. pattern of the serum from that seen in the germfree animal. Monocontamination with *Streptococcus faecalis* resulted in agglutinating antibody titers of the same order as that found in the conventional animal against this species, but precipitating antibodies against one of the cell constituents, the Lancefield group D polysaccharide, could not be demonstrated. It was only when the contaminating organisms were also administered intraperi-

toneally (see Wagner, elsewhere in these pages) that higher than germfree globulin levels were found in many instances, restricted for the most part to an increase in the gamma fraction. However, agglutinating antibody titers remained at the same level as before. Normal monocontamination apparently gives rise to relatively small amounts of high-grade agglutinins in the circulation but, upon extensive bacterial penetration, other "antibodies" can be formed that then cause a rise in the gamma-globulin fraction. It was for this and other reasons



FIGURE 3. Immunoelectrophoretic patterns indicating true gamma globulin in germfree rat serum (see text). In wells: (*left*) conventional rat serum; (*middle*) rat gamma globulin; (*right*) germfree rat serum. In troughs: (*left*) anticonventional rat rabbit serum; (*middle*) antigermfree rat rabbit serum; (*right*) anticonventional rat rabbit serum.

that we were interested in determining how the germfree rat would react to an even more simple antigenic stimulus: the administration of bovine serum albumin (BSA). As soluble or alum-precipitated BSA produced no perceptible response in the Lobund rat, Freund's incomplete adjuvant (FIA) was added to the antigen to increase the response of the animal without adding more antigenic material.

Animals in series I received 2 doses of BSA spaced at 2-month intervals. Each injection consisted of 5 mg. BSA-nitrogen given intraperitoneally and the balance subcutaneously in the left and right inguinal region for a total dose of 30 mg. BSA-N per kg. body weight. Where FIA* was used, it was mixed 1:1 with the BSA solution. In series II, BSA was administered with or without FIA in 3 mounting doses given at weekly intervals. The doses, given in terms of total BSA protein were: first dose, 10 mg. intraperitoneally; second

TABLE 1
GLOBULIN FRACTIONS* AND ANTIBODY IN SERUM OF GERMFREE, BSA-TREATED,
GERMFREE, AND MONOCONTAMINATED ADULT LOBUND RATS

	Beta	Δ †	Gamma	Δ	Antibody
GF	299	—	126	—	—
BSA	314	0/6	129	0/6	0/4‡
FIA	333	1/9	150	1/9	0/4‡
BSA + FIA I.	909	2/2	207	1/2	—
II.	482	4/6	172	2/6	5/6‡
<i>S. faecalis</i> (mono + l.p.)	377	1/4	241	3/4	4/4§
Conv.	591	11/12	523	12/12	12/12§

* Average values in mg. per cent.

† Animals outside the range of germfree serum values.

‡ Anti-BSA precipitins detectable with gel-diffusion technique.¹⁴

§ Anti-*S. faecalis* agglutinins present.

dose, 15 mg. in the scruff of the neck; third dose, 20 mg. in left inguinal region. The animals in both series were sacrificed 3 weeks after the last injection.

As expected, the results show that only when BSA + FIA are used in combination did a perceptible reaction (TABLE 1) occur. In contrast to the reaction to bacterial invasion, the change in the F.B. pattern is now mostly in the beta range. Compared to the rise in serum globulins the precipitin formation, though unmistakable, is very slight. Using the technique recently published by Osserman,¹⁵ we find that the greater part of this anti-BSA precipitating antibody is located in what is usually considered the gamma region of the agar gel pattern. Even while assuming that part of these anti-BSA precipitins are actually located in the beta globulin fraction, a great part of the beta globulin increase seen in the F.B. patterns (on the average 2 mg./ml.) must represent protein of incomplete or no anti-BSA specificity. This situation might be comparable to the 4 to 5 mg./ml. rise in gamma globulin seen in chickens

* Freund's Incomplete Adjuvant, Difco Laboratories, Detroit, Mich.

due to the presence of *S. faecalis*, while the serum has only fair agglutinating capacity (Wagner, elsewhere in this publication).

Gamma Globulin in Rabbits and Rats

It is obvious that if the above experiment could have been done in germfree rabbits, we should have expected, offhand, different results. Thus far, however, germfree rabbits are available in limited quantities only. The present germfree rabbit still has an enlarged cecum (unless ligated early in life), but otherwise grows well and has an acceptable appearance. However, no young born from germfree mothers has as yet survived, and therefore all available animals unhave been obtained by cesarian section and have gone through the rather natural handfeeding period before they were weaned to solid diets.¹⁶ Survival during the handfeeding period is now better than 80 per cent.

When on the basis of the F.B. pattern of rabbit serum a rough separation is made into albumin, alpha, beta, and gamma globulin, then only the gamma fraction is affected by the presence or absence of the flora: gamma globulin

TABLE 2
SERUM PROTEINS OF GERMFREE AND CONVENTIONAL RABBITS*

Age: 3 to 4 months

Diet: L-461-E₃

	Alb.	α	β	γ	Total
Germfree (5)	3280 \pm 125	1194 \pm 96	1303 \pm 100	118 \pm 15	5850 \pm 151
Conv. (6)	3667 \pm 127	1213 \pm 86	1282 \pm 68	823 \pm 46	6985 \pm 268
Rel. mobility (range)	100	85-65	55-39	26-19	

* Free-boundary electrophoresis technique. Values in mg. %. S.D.M. values given.

being all protein with a relative mobility of 30 or less, albumin being 100 (TABLE 2). Most of the germfree rabbit sera available thus far have shown a trace of gamma-globulin, from 0 to 2 per cent, against 10 to 12 per cent in the sera of conventional animals. The cesarian-born germfree rabbit is born with an ample supply of maternal prenataally transferred gamma-globulin. As there is no colostrum transfer, this gamma-globulin disappears rapidly. In the presence of a microflora it takes about 4 to 5 weeks before the animal's own gamma-producing mechanisms take over to a perceptible extent and the gamma fraction again increases (FIGURE 4). In the absence of a flora, a low minimum is reached and, in so far as we know, maintained. Compared to the rabbit, the conventional rat is born with little gamma globulin. Taking into account the slow reaction of the gamma globulin producing mechanism of the ex-germfree rat upon exposure to a normal microflora^{2,11} it would seem that the rapid increase indicated by our data is brought about by colostrum transfer rather than by active antibody production. Whatever antibacterial antibody is produced by the rat in the very first weeks of life seems to be concentrated mainly in the beta-globulin fraction.⁷ The germfree rat, in this case suckled by its germfree mother, stabilizes at the approximate 2 per cent gamma-globulin level also found in the serum of the mother animal. This percentage is es-

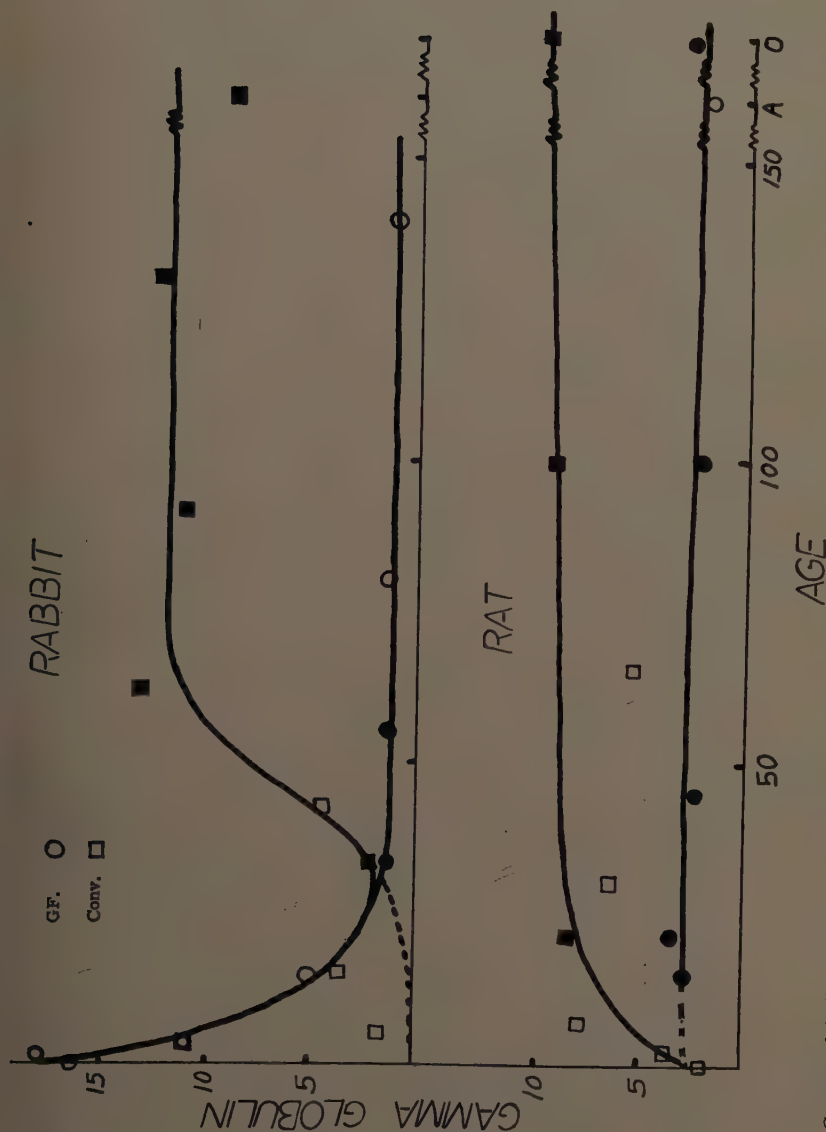


FIGURE 4. Gamma globulin of germfree and conventional rats and rabbits with increasing age. Solid symbols indicate three or more observations. A = adult; O = old.

tablished in the suckling stage and, on the rat diets presently in use, is still roughly the same at the age of 18 months.

It is difficult to speculate as to the nature of the gamma globulins found in germfree animals. The present germfree animal still is exposed to a number of antigenic stimuli not derived from viable microorganisms. Wagner has shown that older germfree chickens show positive agglutination titers against *Micrococcus epidermidus*, presumably caused by the consistent presence of heat-killed microbial forms in the diet.¹⁷ Moreover, the steam-sterilized diets would be expected to have a certain antigenicity. We have indications that especially the presence of bovine milk proteins in the sterilized diets may add to their antigenic properties. Experiments now in progress with water-soluble, antigen-free diets fed to germfree animals starting from birth should shed some light on this problem.

Guinea Pigs

The effect of diet could also be reflected in the results obtained with germfree guinea pigs. In 1958 I reported (1) that no gamma globulin had been found in 1-year-old germfree guinea pigs reared on diet L-445 (mixture of Purina Lab Chow and Quaker Oats).¹⁸ Lately I have tried to improve the diets, and one of the results seems to be the presence of a fair amount of gamma globulin in these germfree animals. Newton's results show that in the guinea pig, as in the rabbit, the prenatally obtained gamma-globulin level declines at first,¹⁹ although apparently at a slower rate, which indicates simultaneous new formation during that period. Both germfree and conventional animals show a similar decline and then reach a low of approximately 100 mg. per cent. Only after the age of 8 to 10 weeks does the presence or absence of a microflora seem to make itself felt. (J. Tanami, personal communication.) My own data essentially confirm these findings. The impression is created that the gamma fraction in the guinea pig reacts only slowly to the presence of a microflora and that in this case other factors are more prominent in stimulating gamma-globulin formation than in other species. This could possibly explain the fact that, thus far, we have not succeeded in bringing the germfree guinea pig out of the germfree environment and have it survive (in contrast to rabbits and rats). The possible importance of the diet factor is given by the data in TABLE 3, which seems to show that, except for the closely related ruminants, bovine milk protein in the diet could be a stimulant for gamma-globulin formation.

Ruminants

In ruminants no prenatal transfer of immune globulins occurs, and the cesarian-born animals brought up without the mother have essentially no gamma globulin detectable by free-boundary or gel-electrophoresis techniques (TABLE 4). The young goats and sheep in the isolators were fed a fortified milk formula. The goats, so far as C. K. Smith* followed them (130 days), were healthy and took the diet well. In sheep, after the first 2 months, the gain in weight slowed down and, at autopsy, calcium deposits were found in the heart, together with a fatty liver and rather bad kidneys.

* Then in residence at Lobund Laboratories, now at the Department of Microbiology, Michigan State University, East Lansing, Mich.

The serum of the germfree goat at 100 days of age (on this diet) was still devoid of gamma globulin, but another animal that became accidentally contaminated during the cesarian birth (with a bacillus and a coccus) showed a slow but definite gamma formation, although low in comparison to that of a goat kept under conventional conditions on a hay and protein mixture (FIGURE 5). Separating the serum proteins roughly into albumin, alpha, beta, and

TABLE 3
EFFECT OF BOVINE MILK PROTEIN IN DIET ON LEVEL OF GAMMA
GLOBULIN IN VARIOUS SPECIES*

Species	Diet	Milk protein % of dry diet	Gamma globulin (%)	
			Conv.	GF
Rat	L-356	20	9	2
Rabbit	L-461E ₃	10	12	1
Guinea pig	L-445	1-2	8	0
	L-462 series	18	12	6
Goat (100 days)	Milk formula	27	6	0

* Electrophoresis: free-boundary technique.

TABLE 4
SERUM PROTEINS IN GERMFREE AND CONVENTIONAL GOATS AND SHEEP*

Species	Status	Age	Alb.	α	β	γ
Goat	GF	100 d.	2642	935	1041	0
Goat	Conv.	100 d.	2895	1363	1124	352
Sheep	GF	129 d.	2489	1172	924	0
Sheep (3)	Conv.†	2-5 y.	3153	834	1156	851
Relative mobility (range)			100	88-68	60-36	30-11

* Free-boundary electrophoresis technique. Values in mg. per cent. Diet: fortified milk formula unless indicated.

† Practical-type diet.

gamma globulin, it seems that both in the germfree sheep and in the germfree goat only the gamma fraction is deficient.

Conclusions

Depending on the species, the present germfree animals show a deficiency in one or more globulin fractions. Studies in the rat show that essentially all protein entities discernible with immunoelectrophoresis techniques in the conventional animal are also present in the germfree, although especially the amount of gamma globulin is strongly reduced. In all species studied thus far, analysis of the serum of the germfree animal and also its reaction to antigenic stimuli point to the gamma-globulin fraction as the main antibody-containing fraction. The capacity to form antibodies seems essentially un-

100-day conv.

90-day cont.

15-day cont.

100 day GF.



FIGURE 5 Agar gel electrophoresis of the serum of a germfree, a contaminated and a conventional goat (see text).

impaired in the germfree-born animal. Antigenic stimulus will at times result in a response that seems to indicate the formation of considerable amounts of protein with incomplete or no antibody specificity.

Acknowledgments

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Part V. Influence of Nonspecific and Physiologic Conditions Affecting the Plasma Proteins

SERUM GLYCOPROTEINS IN THE RHEUMATIC DISEASES

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The dynamic changes induced by inflammation in those carbohydrate moieties intimately associated with serum protein have been described in several excellent reviews.¹⁻⁴

Inflammatory responses are fundamental in the pathology of the rheumatic diseases and the classification of these diseases is partially based on particular tissues involved, local intensity and degree of evoked systemic reaction.

A variety of laboratory measures are applicable to the detection and quantitation of systemic inflammatory response. These 'acute phase reactants'⁵ include the erythrocyte sedimentation rate,⁶ C-reactive protein,⁷ seromucoid,⁸ serum sialic acid,⁹ serum protein fractions,¹⁰ and serum glycoprotein as determined either by hexosamine¹¹ or hexose¹² content. These acute phase reactants measure similar phenomena although there are differences in the accuracy with which these various tests quantitate the wide range of inflammatory response. The work of my associates and myself has centered around serum glycoprotein determined as the hexose. It has been our experience that total serum glycoprotein changes generally become more meaningful when related to the associated total serum protein.¹³ We have expressed this product¹⁴ as the polysaccharide-protein ratio (PR).^{*} The present report is concerned with characteristics of PR changes in various of the rheumatic diseases and applications of this measure to rather practical aspects of the diagnosis and management of these diseases. Methods used in deriving the present data have been published in detail.¹⁵

Total Polysaccharide-Protein Ratio in Various Conditions

Characteristics of total PR in various age groups and in several of the rheumatic diseases are summarized in TABLE 1. While total serum protein is low during the first three months of life¹⁶ serum glycoprotein is comparatively slightly lower, as reflected in the low ratio seen also in adult individuals with impairment of liver function.⁴ Approximately normal adult PR values are found in the age groups three months to three years, although a relatively large variation among these individuals occurred in our series. Adult PR values in the absence of liver disease, infection, fever, cancer, or pregnancy remain within relatively normal limits and without appreciable sex difference. Very old individuals tend to develop a slight elevation of PR, possibly as the result of incipient pathological processes common to these individuals.

Patients with psychogenic rheumatism exhibit normal PR values. The

^{*} $\frac{\text{Total serum protein-bound polysaccharide (hexose) in mg./100 ml.}}{\text{Total serum protein in gm./100 ml.}} \times 100 = \text{PR.}$

slight increase in PR provoked by degenerative joint disease is consistent with the relatively mild inflammatory manifestations frequently accompanying this form of arthritis. True rheumatoid spondylitis characteristically induces only modest elevations of PR; however, extension of the disease to peripheral joints is accompanied by a greater rise in PR.

Inactive phases of rheumatic fever may be readily distinguished from active stages of the disease by means of the PR and with the same degree of accuracy by glycoprotein alone.¹⁷ Although the PR changes rather slowly in response to changes in disease activity and may tend to delay ambulation, it serves as a sensitive and dependable index of rheumatic fever activity.

TABLE 1
POLYSACCHARIDE-PROTEIN RATIO IN VARIOUS OF THE RHEUMATIC DISEASES

	Number of patients	Average PR* \pm S.E.
Normal		
0-3 mo.	8	1.54 \pm 0.04
3 mo.-1 yr.	6	1.82 \pm 0.08
3 yr.-12 yr.	21	1.63 \pm 0.02
Adult	28	1.71 \pm 0.02
Aged 70+ years	15	1.79 \pm 0.02
Psychogenic rheumatism	3	1.72
Degenerative joint disease	28	1.85 \pm 0.02
Rheumatoid spondylitis	15	1.88 \pm 0.09
Rheumatic fever		
Inactive	15	1.91 \pm 0.06
Active	18	2.78 \pm 0.10
Rheumatoid arthritis		
Inactive	8	1.91 \pm 0.03
Mild activity	13	2.26 \pm 0.06
Moderate-severe activity	12	2.73 \pm 0.06
Gout		
Inactive	8	1.96 \pm 0.09
Active	5	2.70 \pm 0.04
Dermatomyositis	4	2.10 \pm 0.03
Scleroderma	11	2.24 \pm 0.08
Periarteritis nodosa	4	3.05 \pm 0.12
Systemic lupus erythematosus	13	3.05 \pm 0.05
Juvenile rheumatoid arthritis	31	2.62 \pm 0.10

* Polysaccharide-protein ratio.

Rheumatoid arthritis can be ordinarily differentiated with ease by gross clinical observation into active and inactive stages. However, accurate and reproducible clinical quantitation of rheumatoid activity is difficult and must take into account the variety of systemic manifestations of the disease as well as localized joint manifestations. A simple system of classifying rheumatoid disease into degrees of clinical activity is presented in TABLE 2.¹⁸ The PR shows excellent correlation with the degree of rheumatoid arthritis and, in our hands, has been more expressive in this regard than other acute phase reactants with which it has been compared.¹⁹

A tendency to slight elevation of PR occurs in interval gout, particularly if tophaceous, while more florid changes follow attacks of acute gouty arthritis or develop during clinically active phases of the disease.

There is great variation in the degree of systemic inflammation, as manifested by the PR, between individuals with scleroderma or dermatomyositis; while periarteritis nodosa, systemic lupus erythematosus, and juvenile rheumatoid arthritis are generally characterized by comparatively high elevations of PR. While we have not carefully correlated PR with intensity of the disease process in the latter conditions it is our impression that, as in rheumatoid arthritis, the

TABLE 2

ESTIMATION OF INFLAMMATORY ACTIVITY IN PATIENTS WITH RHEUMATOID ARTHRITIS

Clinical activity	Morning stiffness	Sleep pattern	Malaise, fatiguability, and weakness	Acute joint manifestations	Fever	Appetite	Physical accomplishments
I (None)	None	Undisturbed	Absent	Absent	None	Good	Limited only by deformity
II (Mild)	10 min.	Undisturbed	Slight	Transient joint pains	None	Untroubled	Limited only by fatiguability and deformity
III (Moderate)	<30 min.	Mild nocturnal restlessness; early morning awakening	Moderate	Joint swelling without redness or heat	None or slight temp. elevation	Capricious	Limited by joint pain, fatiguability, and deformity
IV (Severe)	>30 min.	Frequent awakening (fatigue)	Pronounced	Joint swelling with redness and heat	Elevation of body temp.	Anorexia	Limited by general malaise, joint pain, fatiguability, and deformity

TABLE 3

CHANGE IN PR RELATED TO CLINICAL CHANGE IN ACTIVITY OF RHEUMATOID ARTHRITIS

Change of 10% or more		
Decrease in PR with clinical improvement	53/67	(79%)
Decrease in PR with clinical exacerbation	3/67	(4%)
Increase in PR with clinical improvement	0/67	
Increase in PR with clinical exacerbation	29/38	(76%)
Change of less than 10%		
Decrease in PR with clinical improvement	10/67	(15%)
Decrease in PR with clinical exacerbation	1/67	(1%)
Increase in PR with clinical exacerbation	9/38	(24%)

PR reflects the degree of systemic inflammation provoked by these diseases and may aid in estimating the prognosis of individual cases.

Relation of polysaccharide-protein ratio to changes in rheumatoid activity. Changes of PR clearly reflect clinical alteration in the activity of rheumatoid arthritis.¹⁴ A high degree of correlation is observed between a decrease in PR with clinical improvement and the converse of an increase in PR with clinical disease exacerbation: if such evidence is based on PR changes of 10 per cent or more (TABLE 3). This margin is comfortably in excess of our maximum technical error of 5% for the combined procedures.

Arthritis in swine and polysaccharide-protein ratio. Experimental arthritis

induced in swine by reaction to the microorganism *Erysipelothrix rhusiopathia*²⁰ has been studied insofar as accompanying changes in acute phase reactants.²¹ While high elevations of PR occur soon after intravenous challenge of the pig with the pathogenic organism, even higher levels are reached during the phase of subsequent arthritis. During inactive phases of the arthritis, PR values

TABLE 4

POLYSACCHARIDE-PROTEIN RATIO IN SWINE ERYSIPELAS AND SUBSEQUENT ARTHRITIS

	Number of animals	Average PR \pm S.E.
Normal adult swine	11	2.27 \pm 0.06
Initial infection	16	2.85 \pm 0.06
Active arthritis	12	3.10 \pm 0.06
Inactive arthritis	4	2.28

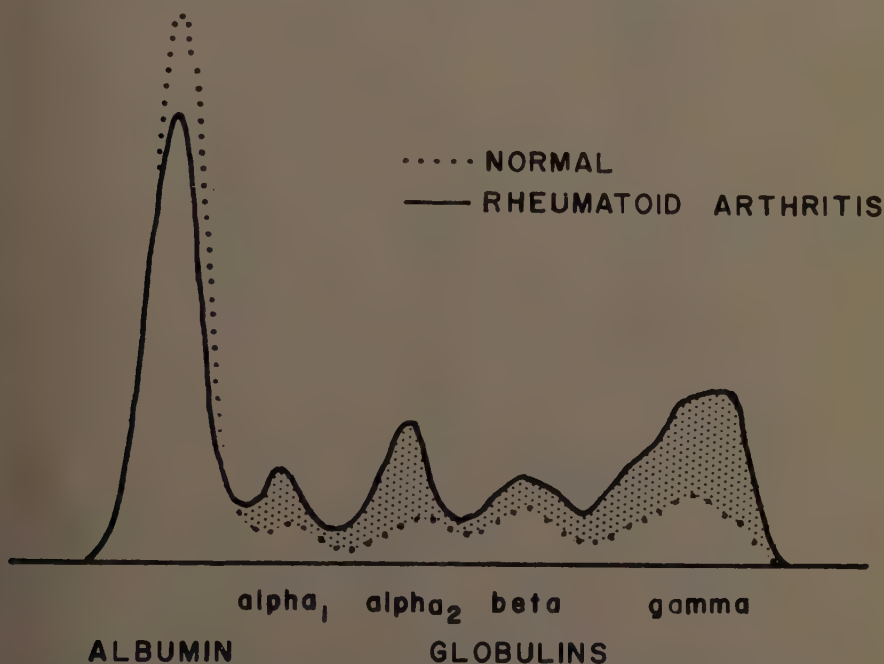


FIGURE 1. Separation of protein components of normal and rheumatoid sera by electrophoresis in barbital buffer.

return to normal (TABLE 4). It is of interest that although α_1 globulin and glycoprotein are not clearly discernible in adult pig serum, substantial amounts of seromucoid are present, increasing in proportion to the degree of inflammatory response.²¹

Electrophoretically separated rheumatoid serum components. Studies of electrophoretically separated protein and glycoprotein moieties allow close inspection of the interplay of factors contributing to gross changes in these components.

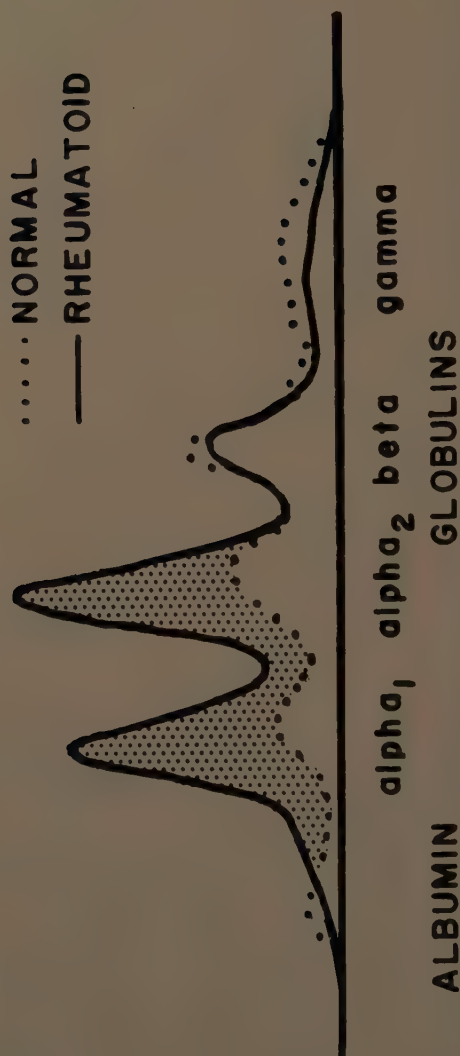


FIGURE 2. Separation of glycoprotein components of normal and rheumatoid sera by paper electrophoresis in barbital buffer.

Densitometer tracings of normal and rheumatoid sera electrophoretically separated on filter paper in barbital buffer (pH 8.6; 0.075 M) are compared when stained for protein (FIGURE 1) and glycoprotein (FIGURE 2). Decrease in serum albumin and increase in globulin fractions are observed in active rheumatoid arthritis.^{10,13} In this disease the abundant increase in protein-bound carbohydrate associated with the alpha globulins is apparent.¹⁵

In order to better visualize quantitative aspects of electrophoretically separated patterns we have utilized the bar graph. Each protein and glycoprotein

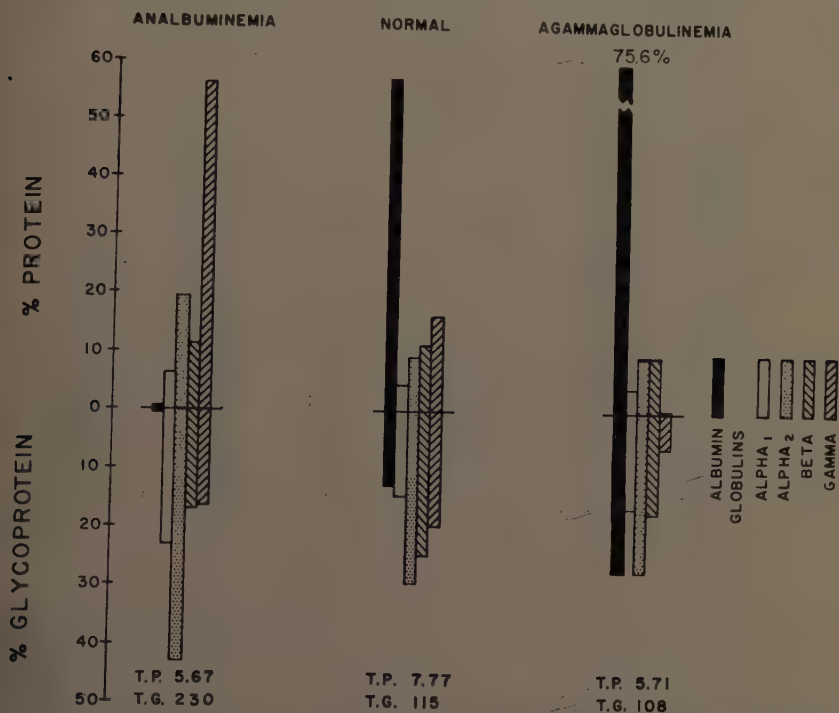


FIGURE 3. Representation of protein and glycoprotein distribution by paper-strip electrophoresis in the normal individual and in 2 patients with striking deviations from the normal. The total concentration of serum protein (gm./100 ml.) and serum glycoprotein (mg./100 ml.) is indicated beneath each graph.

fraction is plotted as the percentage that it contributes to the total protein or glycoprotein. In order to appreciate the true amounts of glycoprotein it must be kept in mind that the relative proportion of glycoprotein must be divided by a factor of approximately 75 to bring it into quantitative perspective with the carrier protein.

Normal protein and glycoprotein fractions are compared to those of a patient with analbuminemia and one with agammaglobulinemia in FIGURE 3. The analbuminemic, who is also a rheumatoid arthritic, shows elevated alpha globulin proteins with a commensurate increase in associated glycoprotein, accounting for the greater part of his bound carbohydrate.²² Though this

individual exhibits relatively large amounts of gamma globulin only a small proportion of carbohydrate is bound in this region. The agammaglobulinemic child, who does not show evidence of rheumatic disease, distributes a relatively great portion of protein in the albumin region and appears to carry an appreciable amount of carbohydrate with his minuscule amounts of gamma globulin.

Comparisons of the various fractions of protein and glycoprotein in patients with rheumatoid arthritis of various degrees of clinical activity compared to

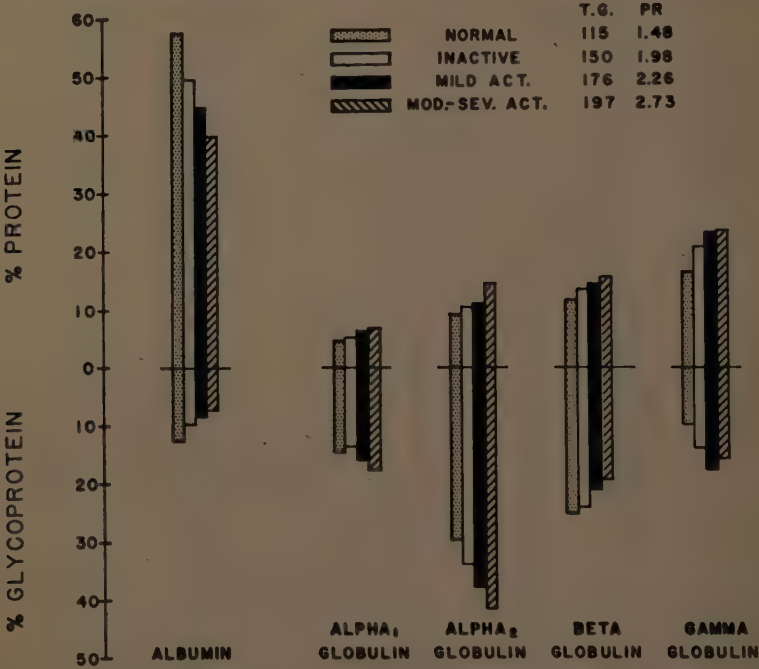


FIGURE 4. Comparison of the average concentration of serum protein and glycoprotein components, as separated by conventional paper electrophoresis, in rheumatoid arthritis of several grades of severity compared to the normal individual.

normal individuals are presented in FIGURE 4. The decline in albumin and increase in the alpha globulin proteins and glycoproteins associated with increasing activity of rheumatoid arthritis is evident. Beta globulin tends to increase slightly with disease activity although it appears to be progressively robbed of its carbohydrate moieties by other fractions. Gamma-globulin protein and glycoprotein show relatively poor linear response to increasing activity of the disease.

Correlation of the above observations with clinical activity of rheumatoid disease yields the conclusions of TABLE 5. Total PR exhibits better correlation with clinical rheumatoid activity than any of its component fractions or than seromucoid, C.R.P., or serum sialic acid.²³

While abundant changes in α_2 protein and glycoprotein occur in rheumatoid arthritis, the PR of these components relatively poorly expresses rheumatoid activity (TABLE 6), undoubtedly due to a proportionate increase in both moieties. A similar finding, determining polysaccharide as hexosamine, has been reported.²⁴ While α_1 protein does not accurately reflect rheumatoid activity, α_1 glycoprotein (which contains the greater part of seromucoid) is closely associated with this process.

TABLE 5

CORRELATION OF SERUM PROTEIN AND PROTEIN-BOUND CARBOHYDRATE COMPONENTS WITH CLINICAL ACTIVITY OF RHEUMATOID ARTHRITIS*

	Correlation coefficient*
Total PR	0.914
α_1 PR	0.559
Serum glycoprotein	0.590
Seromucoid	0.689
Albumin protein	-0.563
α_2 protein	0.635
α_2 glycoprotein	0.684
α_1 glycoprotein	0.654
Sialic acid	0.588
C-reactive protein	0.515

* Significant at the 1 per cent level.

TABLE 6

CORRELATION OF α_1 AND α_2 PROTEIN AND GLYCOPROTEIN MOETIES WITH CLINICAL ACTIVITY OF RHEUMATOID ARTHRITIS AND TOTAL POLYSACCHARIDE-PROTEIN RATIO

	Correlation coefficient	P
α_1 protein \times clinical activity	0.110	0.10
α_1 glycoprotein \times clinical activity	0.654	0.01
α_1 PR* \times clinical activity	0.559	0.01
α_1 PR \times total PR	0.686	0.01
α_2 protein \times clinical activity	0.635	0.01
α_2 glycoprotein \times clinical activity	0.684	0.01
α_2 PR \times clinical activity	0.296	0.10
α_2 PR \times total PR	0.706	0.01

* Polysaccharide-protein ratio.

Serum changes induced by prolonged glucocorticoid administration to patients with rheumatoid arthritis, to the end point of readily apparent Cushingoid features, are compared to those of the relatively rare rheumatoid who has never received glucocorticoids. No substantial difference as to serum protein or glycoprotein components was noted in these patients, although our data are consistent with reports of a slight decrease of gamma globulin in rheumatoids treated with glucocorticoids,^{25,26} and we have noted a similar effect in arthritic pigs treated with cortisone.²¹

Comparison of glucocorticoid and nonglucocorticoid-treated rheumatoids

with a series of juvenile rheumatoids treated by various means reveals a small but significant additional increase in the gamma globulin of these relatively sick little individuals (FIGURE 5).

Electrophoretically separated myeloma serum components. Six cases of multiple myeloma have been followed electrophoretically and representative patterns are compared in FIGURE 6. Our cases all exhibit relatively abundant bound carbohydrate on the myeloma protein although there is a tendency for

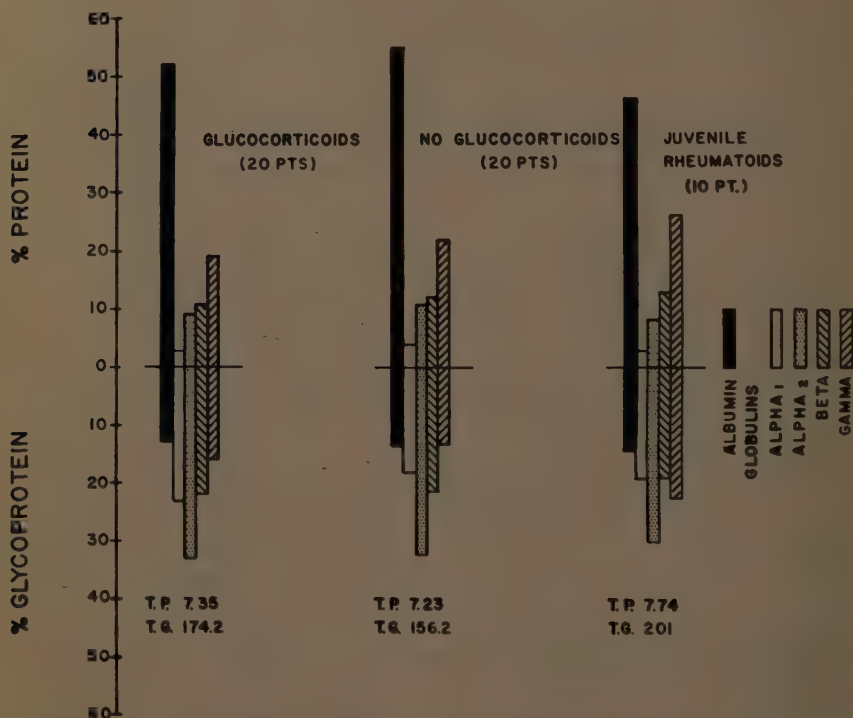


FIGURE 5. Comparison of average concentrations of serum protein and glycoprotein components, as separated by conventional paper electrophoresis, in juvenile rheumatoid arthritis and in glucocorticoid and nonglucocorticoid-treated rheumatoid arthritis.

myeloma protein of the beta and "fast" gamma type to be particularly rich in bound carbohydrate, as reported by Nikkila.²⁷

Evaluation of response to treatment with polysaccharide-protein ratio. Routine use of the total PR has been applied to objective evaluation of the response of rheumatoid patients to various medications (TABLE 7). Vagaries in subjective evaluation of clinical response in patients with rheumatoid arthritis are well known, as illustrated by the response of 50 per cent of such patients to oral placebo as reported in one study.²⁸

Our studies using the PR as an integral factor in evaluation of rheumatoid activity have included serial determination of this measure every 2 to 4 weeks.

It is our present impression that a minimum treatment period of 4 weeks is optimal for equilibration of PR to a significant change in disease activity.

When judged on the basis of PR, placebo response is quite low: it is similar to the incidence of unexplained decrease in PR with clinical exacerbation

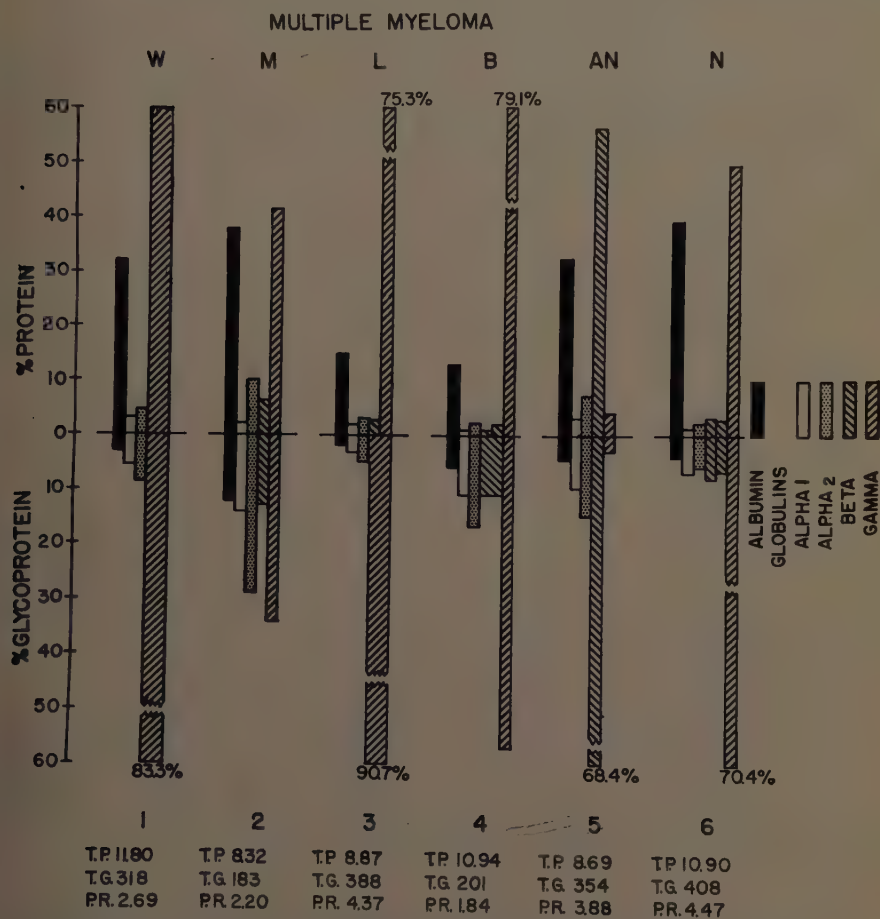


FIGURE 6. Comparison of serum protein and glycoprotein moieties, as separated by conventional electrophoresis, of sera from patients with multiple myeloma.

(TABLE 3). Potent anti-inflammatory properties of phenylbutazone and various of the glucocorticoids, as reflected in PR changes, are illustrated in TABLE 6.²⁹ Indeed, the relative antirheumatic potency of the various glucocorticoids can be assayed by this means.

Aspirin, in the doses used, proved a relatively weak anti-inflammatory agent as measured by PR changes and no synergism between small doses of glucocorticoids and aspirin could be demonstrated by this measure.³⁰

Relatively long periods of observation are required to demonstrate the antirheumatic effect of chloroquine. This agent in a single dose of 250 mg. daily produced significant reduction of PR in 41 per cent of rheumatoids after continued administration for a period of 3 months. Larger doses of chloroquine (250 mg. B.I.D.) induced a drop in PR after a minimum treatment period of 6 weeks though no greater percentage of patients showed improvement by this measure.

TABLE 7
SIGNIFICANT CHANGES OF POLYSACCHARIDE-PROTEIN RATIO* IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED WITH VARIOUS MEDICATIONS

Drug	Daily dose (mg.)	Decrease in PR†
Phenylbutazone	50-100	5/14 (36%)
	200-300	27/39 (69%)
	301-400	24/27 (89%)
	401-600	2/2
Cortisone acetate	50-100	8/21 (38%)
	101-200	7/9 (78%)
Prednisone	5-10	3/15 (19%)
	15-20	3/14 (27%)
	25-30	6/14 (46%)
Prednisolone	5-10	4/11 (27%)
	15-20	7/26 (46%)
	25-30	8/11 (67%)
	40	1/1
Aspirin	2400	8/48 (16%)
Aspirin + prednisolone	2400 + 8	5/31 (16%)
Chloroquine phosphate	250	7/17 (41%)
	500	6/16 (38%)
Placebo (lactose)	2400	4/63 (6%)

* Decrease of 10 per cent or more.

† Polysaccharide-protein ratio.

Summary

The processes of inflammation incidental to various kinds of the rheumatic diseases are reflected by dynamic changes in serum glycoprotein concentrations. A particularly meaningful and quantitative expression of these changes is made possible by relating serum glycoprotein concentration to its carrier serum protein (PR). In the normal adult the PR remains within fairly constant limits. Rheumatic diseases induce elevations of PR in proportion to the degree of systemic inflammatory response evoked. Thus degenerative joint disease and rheumatoid spondylitis characteristically cause little increase in PR while systemic lupus erythematosus and periarteritis nodosa are characterized by high PR. The PR is of interest in quantitation of activity of the

more acute rheumatic diseases and by this measure active and inactive phases of rheumatic fever, rheumatoid arthritis, gout, and other rheumatic diseases can be objectively separated. Degree of rheumatoid arthritis activity can be quantitated by this measure, thus affording a measure of the course of the disease. Dose response to various agents used in the treatment of rheumatoid arthritis has been determined by serial PR determinations.

Fractionation of serum protein by paper strip electrophoresis and staining of individual proteins for carbohydrate content have permitted detailed study of the spectrum of glycoprotein changes in rheumatoid arthritis of various degrees of severity, multiple myeloma, analbuminemia, and agammaglobulinemia.

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THE GLYCOPROTEINS AND THEIR RELATIONSHIP TO THE HEALING OF WOUNDS*

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"Mucosaccharides" are widely distributed in tissues and fluids of the body.¹ In plasma there are a number of distinctly different glycoproteins found associated with the globulin fractions.² In connective tissues both acid mucopolysaccharides and soluble glycoproteins are found in the semifluid ground substance between the collagen fibers.³

Ordinarily under physiological conditions, the constituents of connective tissues have a rather low turnover and remain relatively constant. But when abnormal conditions arise, such as an injury, alterations occur. Locally this involves variable damage to tissues and vessels while a systemic response is usually indicated by blood plasma changes.^{4,5} Variations, particularly in the glycoprotein components of plasma, have been observed after wounding and inflammation.⁶

During wound healing, changes occur in concentrations of polysaccharide material of the wound area. An early accumulation of bound hexosamine has been reported in repair tissue⁷ as well as in tissue surrounding the wound.⁸ Increased amounts of acid mucopolysaccharides have been found in proliferating tissue both by chemical analysis⁹ and with tracer studies.¹⁰ The influence of vitamin C on the acid mucopolysaccharide content of the wound has been a subject of wide interest.¹¹ Granulomas produced by carageenin injections in scorbutic animals contain mucopolysaccharides of the hyaluronate type while chondroitin sulfate is depressed.^{10,12,13} Evidence seems to indicate that glycoproteins are involved in the complex processes of growth and repair of connective tissue and that, as such, they are intimately related to wound healing.

The significance of glycoproteins in wound healing has been confused by discrepancies in their nomenclature and classification as well as by variable wounding, tissue sampling, and fractionating methods. Nor has the presence of extracellular fluid been adequately studied. It seems pertinent for the evaluation of our data, therefore, to describe briefly the methods employed in each section of this study.

Methods

The experimental wound. In our studies use of subcutaneously implanted stainless steel wire mesh cylinders has provided a unique experimental model of the biological environment of the healing wound. This method was developed by Schilling *et al.*¹⁴ and subsequently modified for use in dogs and described in greater detail elsewhere.¹⁵ Cylinders such as those shown in FIGURE 1a are implanted beneath the skin of the dog's back for various periods of time before removal. The cylinder becomes encapsulated within the sub-

* The work reported in this paper was supported by the Department of the Army, Research and Development Division, Washington, D.C.

dermal tissue of the host. Beginning about 5 days postwound, a fluid can be aspirated from within the cylinder. The space within the cylinder is gradually filled in with a fibrous connective tissue that assumes the form of a tube (FIGURE 1*b*). This tube, which contains wound fluid at all times, can be removed from the metal cylinder rather easily and has been used for vessel-grafting purposes.¹⁶ This connective tissue has been observed to be deposited in orderly fashion in concentric layers. A cross section of a tube (magnified 9 times) and stained



FIGURE 1. (a) Stainless steel wire mesh cylinder with lucite rings within for support. (b) Magnification of upper one-third section of mesh cylinder opened after 32 weeks' implantation. Note tubular formation of fibrocollagenous tissue, partially separated from the wire mesh.

with osmic acid (FIGURE 2*a*) clearly illustrates this. Each osmiophilic lamella corresponds to a growth period of approximately 3 weeks. A cross section of tissue stained with silver (FIGURE 2*b*) typifies the organization of fibers longitudinally and with radial interlacing of fibers between the lamellae.

The method provides an opportunity to sample both the tissue and fluid from within the wound at any time throughout the course of fibroplasia. The newly formed avascular connective tissue is discretely separated from the old (host) tissue by the wire-mesh barrier. Unlike the results of other techniques no foreign agent is present within the new repair tissue nor has there been any evidence of foreign body reaction such as the presence of giant cells. Also

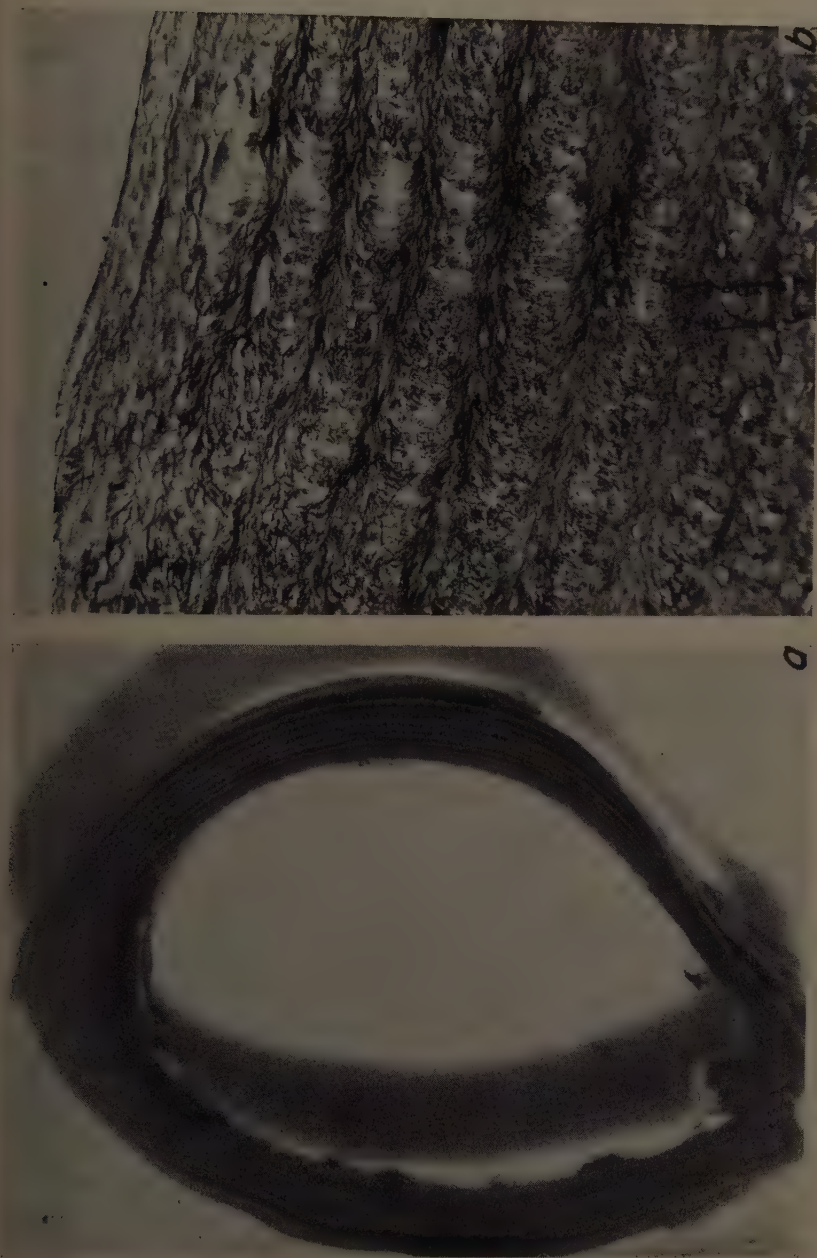


FIGURE 2. (a) Cross section of the fibrocollagenous tube fixed and stained with osmic acid. $\times 9$. Note the regular lamellations. The light bands are the intervening areas of loose interlacing radial collagenic fibers.¹⁶ Reproduced by permission of *A.M.A. Archives of Pathology*. (b) Cross section, silver stained, showing organization of fibers. $\times 250$.

eliminated are infection, epithelialization, and contracture, which commonly complicate wound-healing studies.

Tissue fractionation. At designated times throughout the healing process, cylinders were surgically excised and wound-fluid contents aspirated. Extraneous tissue outside the cylinder was removed, and then the cylinder and inner tissue contents were lyophilized. The dried tissue, stripped by hand from the cylinder, was extracted with ether for 48 hours, and then ground to pass through a 60-mesh screen. This material was extracted with 0.9 per cent NaCl solution for 24 hours. The residue was again extracted for 24 hours with saline and the 2 extracts were combined. The residue was extracted 2 times for 1-hour periods with 0.5 *N* NaOH. After dialysis, the saline and alkaline extracts were each lyophilized. Each lyophilized tissue extract was suspended in water and the pH adjusted with HCl to pH 2. This was digested with pepsin and then precipitated with 4 volumes of ethanol. The precipitate was suspended in a phosphate buffer and subjected to trypsin digestion at pH 7.8 to 8.0. The solution was precipitated with perchloric acid and the residue removed. This supernatant was dialyzed to remove the perchloric acid and then acetic acid was added to make 0.5 *N*. Four volumes of ethanol were added to this mixture. This resulting precipitate was dissolved in 0.1 *N* NaOH and analyzed chemically or utilized for paper electrophoretic studies.

Wound fluid fractionation. Wound fluid was obtained simultaneously with the wound tissue samples along with serum of the animal for comparative purposes. For isolation of the acid mucopolysaccharides, wound fluid and serum were diluted 1:1, heated, and subjected to trypsin and chymotrypsin digestion. Perchloric acid was added to the solution, the precipitate removed, and the supernatant dialyzed to remove HClO₄. Acetic acid was added to 0.5 *N*. Precipitation with 4 volumes of ethanol was followed by dissolving in 0.01 *N* NaOH.

Chemical and electrophoretic studies. Paper strip electrophoretic studies were carried out using Spinco Model R electrophoresis cells, 0.075 *M* barbital buffer, pH 8.6, Whatman 3 MM strips, and current of 5 mAmp. per 8 strips. Staining of strips was done using bromphenol blue for protein, periodic acid-Schiff for glycoprotein, and both toluidine blue and astra blue for acid mucopolysaccharides. Chemical analyses carried out as previously described^{17,18} were: total protein as determined by a biuret method, total protein-bound hexose by tryptophan method, hexosamine by the Elson-Morgan modification by Boas, uronic acid by the carbazole method following preliminary hydrolysis with Dowex 50, and tissue hexose by the anthrone reaction on the hexosamine hydrolysates.

Results

The problems in wound healing in our laboratory have involved: first, the types of mucopolysaccharides and glycoproteins found in repair tissue; second, the changes in types of mucopolysaccharides found in wound tissue during the reparative process; and, third, the relationship of wound fluid and tissue components to wound repair tissue.

It has been found that repair tissue contains a mixture of mucopolysaccha-

rides and glycoproteins¹⁷ as indicated by electrophoretic and chromatographic identification of galactose, mannose, glucose, hexosamines, and uronic acid in the hydrolysates of this tissue. Most of the glucose present may be assumed to be due to glycogen, the galactose and mannose are monosaccharides characteristic of glycoproteins, and uronic acid is probably from the acid mucopolysaccharides.

The changes in uronic acid, hexosamine, and hexose of repair tissue during the healing process¹⁷ are shown in TABLE 1. Uronic acid is low at 7 days and increases to a maximum amount by 35 days. The hexosamine and hexose

TABLE 1
CHANGES IN MUCOPOLYSACCHARIDE COMPONENTS OF WOUND REPAIR TISSUE
DURING HEALING PROCESS¹⁷

Days postwound	Uronic acid	Hexosamine	Hexose
	(mg. per gm.)		
7	0.7	6.8	19
14	1.0	7.1	26
21	1.4	6.2	27
28	1.8	7.6	22
35	2.3	7.2	23
42	2.3	7.3	30

These results are based on analyses of 6 separate samples and calculated as mg./g. of dry lipid-free material.

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TABLE 2
RADIOACTIVITY IN WOUND TISSUE FRACTIONS FROM RATS
INJECTED WITH GLUCOSAMINE-1-C¹⁴

Time	Original dialyzed	Crude mucopoly-saccharide	Crude protein
5 day	1740	173	803
14 day	935	104	383

Figures are in counts per minute per milligram of tissue (M. R. Shetlar, unpublished).

remain rather constant. This may indicate the presence of glycoproteins in the early stages with later formation of acid mucopolysaccharides. As a corollary to this finding, the course of regenerating tissue using a similar wounding technique with rats, and injection of glucosamine 1-C-14 has recently been studied by M. R. Shetlar (unpublished results). Preliminary data shown in TABLE 2 point to a high level of radioactivity in early wound repair tissue. This activity seems to be more closely correlated with the protein-bound carbohydrate rather than the mucopolysaccharide components of the wound.

TABLE 3 contains the results of the chemical analysis of the fractionated tissue.¹⁹ The uronic acid containing mucopolysaccharide extracted by the saline is considered to be mostly hyaluronic acid while that in the alkaline extract is mainly chondroitin sulfate. With this interpretation, then, the

hyaluronic acid may be said to decrease with healing while chondroitin sulfate increases. The small amount of uronic acid in the alkaline extract at seven days is apparently due to incomplete extraction of the hyaluronic acid by the saline solutions.

TABLE 3
URONIC ACID CONTENT OF EXTRACTS OF WOUND REPAIR TISSUE¹⁹

Days postwound	Extracts		Total (mg./gm.)
	Saline (mg./gm.)	Alkaline (mg./gm.)	
7*	0.62	0.26	0.87
21	0.57	1.43	2.00
28	0.38	1.45	1.83
35	0.79	1.03	1.82
42	0.58	1.82	2.40

* One dog for each time period, tissue pooled from 18 implanted cylinders on each animal.

ELECTROPHORETIC MOBILITY,
STAINING WITH ASTRA BLUE

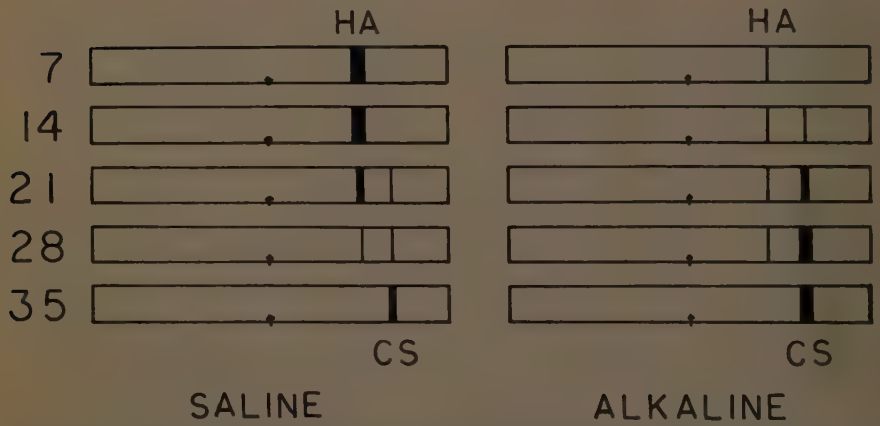


FIGURE 3. Paper electrophoresis studies of the mucopolysaccharides of wound tissue extracts at 7, 14, 21, 28, and 35 days postwound. Extract applied at center of each strip. Mobilities and staining of both hyaluronic acid (HA) and chondroitin sulfate (CS) compared with standard preparations in each experiment.¹⁹

For confirmation of the above, electrophoretic studies¹⁹ of the acid mucopolysaccharides of the repair tissue extracts were carried out and comparisons made to electrophoretic mobilities of standard preparations of chondroitin sulfate (from cartilage) and hyaluronic acid (from vitreous humor). The strips were treated with astra blue (similar to alcian blue), which stains both hyaluronic acid and chondroitin sulfate, and with toluidine blue, which stains sulfated mucopolysaccharides. A graphic representation of the staining with astra blue is shown in FIGURE 3. A single component with the approximate

mobility and staining properties of hyaluronic acid appears at 7 days in the saline extracts. This component decreases in concentration and disappears sometime after 21 days. In the alkaline extracts trace amounts of a second component which appears to be chondroitin sulfate are present at 14 days and increases in staining intensity after that. The staining with toluidine blue agrees with the astra blue staining and with the chemical data in that a greater amount of what may be hyaluronic acid appears in the early wound while chondroitin sulfate predominates in the older tissue.

The relationship of the wound fluid to the tissue components was first studied by chemical analysis of the mucopolysaccharides of fractionated fluid. In TABLE 4 are listed the uronic acid contents of wound fluids.¹⁹ Attempts to demonstrate definite amounts of acid mucopolysaccharide in 1- to 2-ml. samples of serum of wounded animals were unsuccessful, indicating a much lower content of these compounds in serum. The electrophoretic studies of the fluid mucopolysaccharides at various postwound periods in FIGURE 4 reveal

TABLE 4
URONIC ACID CONTENT OF WOUND FLUID¹⁹

Days postwound	Uronic acid (mg./100 ml.)	Protein (gm./100 ml.)
7*	5.4	4.3
14	5.1	4.6
21	7.0	3.8
28	5.9	5.2
35	7.9	4.0
42	7.2	2.9

* One dog for each time period, wound fluid pooled from 18 implanted cylinders on each animal.

an early appearance of a hyaluronatelike material at 7 days that later disappears. A substance with the mobility and staining characteristics of chondroitin sulfate becomes evident at about 21 days.

Comparative studies have also been made of wound fluid and blood serum throughout a 6-weeks postwound period with regard to their protein and glycoprotein content. This work has been done in both puppies²⁰ and adult dogs.¹⁸ In the study shown in TABLE 5, 20 puppies were used. Control serum is that taken from the animals before wounding; postwound serum refers to that obtained during the postwound period; and wound fluid refers to the fluid found within the mesh cylinders during the healing process. The protein values expressed here are averages of all serum and fluid data taken from 1 to 6 weeks postwound. Note here that the electrophoretically defined albumin of wound fluid is elevated while the globulins—alpha 2 and beta 1—are lower as compared to postwound serum values. The increased albumin and decreased globulin of fluid that occurs is thought to be due mainly to permeability and diffusion mechanisms operating in the wound locale. The function of the elevated albumin of the fluid may be to maintain the water content and osmotic pressure of the confined space within the fibrocollagenous tube. More interesting, however, is the distribution of protein-bound carbohydrate or glycoprotein in

serum and wound fluid as shown in TABLE 6 (containing data for the 6 weeks postwound period). Wound fluid has a definitely higher concentration of hexose-containing carbohydrate (expressed as per cent of protein) bound to the globulin fractions than does serum. The significantly elevated glycoprotein fractions are: alpha-1, alpha-2, and beta-1 globulins.

ELECTROPHORETIC MOBILITY,
STAINING WITH ASTRA BLUE

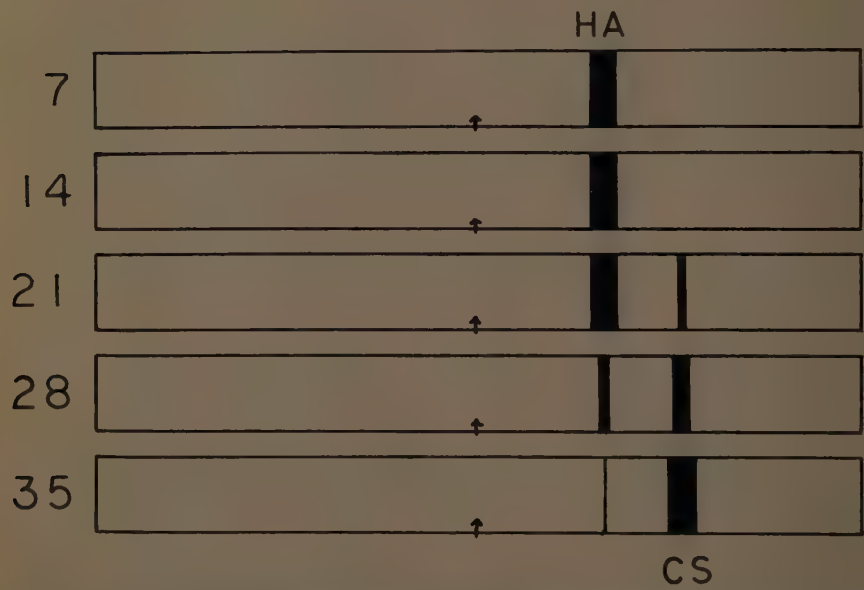


FIGURE 4. Paper electrophoresis studies of mucopolysaccharides of wound fluid extracts at 7, 14, 21, 28, and 35 days postwound. Extract applied at the center of each strip. Mobilities and staining of both hyaluronic acid (HA) and chondroitin sulfate (CS) compared with standard preparations in each experiment.¹⁹

TABLE 5
ELECTROPHORETIC DISTRIBUTION OF PROTEINS IN CONTROL BLOOD SERUM AND IN SERUM AND FLUID COLLECTED FROM WOUNDED PUPPIES DURING A 1- TO 6-WEEK POSTWOUND PERIOD²⁰

Group	Pups	Total protein (gm. %)	Alb. (%)	% Globulins				
				Alpha ₁	Alpha ₂	Beta ₁	Beta ₂	Gamma
Control serum	20	4.7	44.8	12.6	11.6	13.0	14.3	3.7
Postwound serum	20	5.0	41.1	10.2	15.0	15.1	13.9	4.9
Wound fluid	20	2.4*	46.8*	10.7	11.2*	12.1*	14.6	4.7

* Significantly different from postwound serum.

Discussion

The origins of the mucosaccharide components found associated with the reparative process have not been definitely established. Shetlar reports elsewhere in these pages that the synthesis of some of the serum glycoproteins occurs in the liver. Whether the glycoproteins of extracellular fluid arise from liver synthesis via the serum or whether they are formed locally by tissue proliferation or destruction is not known. It seems to be agreed that the fibroblast is the cell responsible for the formation of both the ground substance and collagen fibers. Apparently the various components utilized by the proliferating cells may have different origins.

That some serum glycoproteins are utilized by growing tumor cells in the rat has been demonstrated by Kent and Gey.²¹ Growth-promoting activity by acid mucopolysaccharides on human carcinoma cells has also been reported.²² The possibility that mucopolysaccharides play a major role in the morphogenesis

TABLE 6
ELECTROPHORETIC DISTRIBUTION OF GLYCOPROTEINS IN CONTROL BLOOD SERUM
AND IN SERUM AND FLUID COLLECTED FROM WOUNDED PUPPIES
DURING A 1- TO 6-WEEK POSTWOUND PERIOD²⁰

Group	Pups	Total gly. (mg. %)	Bound hexose* associated with						
			Aib.	Globulins					Total
				Alpha ₁	Alpha ₂	Beta ₁	Beta ₂	Gam- ma	
Control serum	20	91	0.6	4.8	3.9	2.3	1.7	3.4	2.0
Postwound serum	20	104	0.5	5.1	4.2	2.4	1.9	2.8	2.1
Wound fluid	20	54†	0.7	6.6†	5.4†	3.4†	2.1	2.7	2.4†

* Expressed as per cent of the protein moiety.

† Significantly different from postwound serum.

of collagen has been discussed repeatedly.²³ The work of Gross and his associates^{24,25} on the *in vitro* precipitation of collagen by chondroitin sulfate and acid mucopolysaccharides suggests that mucopolysaccharides may be the *in vivo* agents necessary for collagen fibril formation.

The synthesis of collagen and growth of connective tissues, in general, is dependent upon a number of factors including: growth, age, hormones, and nutrition. Vitamin C seems to be especially necessary for the synthesis of collagen but may not be for maintenance. It has been suggested¹¹ that ascorbate is required for the esterification step of chondroitin to produce chondroitin sulfate.

Our work seems to indicate changes in glycoprotein and acid mucopolysaccharide content of both repair tissue and the fluid bathing the interface of fibroplastic response. Although we have progressed beyond the early morphological studies of wound healing, we are still in the descriptive biochemistry phase. Our knowledge of the appearance, increase, and decrease of various components is accumulating; but the mechanisms involved in the regenerative process have yet to be clearly defined.

Summary

Research in the area of wound healing has been hampered by lack of good methods for observing and evaluating the regenerative processes. Using the stainless steel cylinder implant system, the reparative processes have been followed morphologically and by serial chemical studies of fractionated tissue and wound fluid. It appears that glycoproteins predominate in early wound tissue, then acid mucopolysaccharides appear, first as nonsulfated hyaluronic acidlike complexes and then as chondroitin sulfate. Both acid mucopolysaccharides and glycoproteins are found in appreciable amounts in the extracellular fluid in the wound area. Although they are of probable importance in the healing process, no specific role can be assigned to these various mucosaccharides at this time. It is generally agreed that, as essential components of the interfibrillar ground substances, they are vitally linked to the process of collagen fiber formation.

More research utilizing newer tissue sampling methods in combination with tracer studies should clarify much of the process of regeneration of tissue and wound healing.

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SERUM PROTEINS, LIPOPROTEINS, AND GLYCOPROTEINS IN MUSCULAR DYSTROPHY AND RELATED DISEASES*

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Biochemical changes in various forms of muscular dystrophy have been summarized in several reviews.^{1,2} They include changes in serum proteins studied in the hope that they might reflect primary metabolic changes and thus reveal underlying causes of the disease. In the past most of the biochemical studies had been made on an experimental form of myopathy that develops in many species of animals when they are deprived of vitamin E. The symptoms of this form of nutritional muscular dystrophy resemble in many ways those of patients with muscular dystrophy, but they regress when the vitamin is administered.³⁻⁹ This report deals with our comparative studies of the serum proteins, lipoproteins, and glycoproteins in myopathy of vitamin E deficiency, in clinical muscle diseases, and in hereditary muscular dystrophy in mice.¹⁰⁻¹²

Experimental

Muscular dystrophy of vitamin E deficiency was produced in young New Zealand rabbits, as described elsewhere.³

The largest group of patients comprised children with pseudohypertrophic muscular dystrophy (PMD). Other muscle diseases investigated were myositis, myotonia dystrophica, and amyotrophic lateral sclerosis. Some patients with scoliosis and with Perthes' disease also were studied, since sometimes muscular wasting of secondary nature was present.

Dystrophic mice and their control littermates of strain 129† were maintained on Purina Laboratory Chow and sacrificed at ages of 6 to 9 weeks.

The serum proteins, lipoproteins, and glycoproteins in barbiturate buffer of pH 8.6 and ionic strength 0.1 were fractionated by both moving-boundary and paper-electrophoretic methods, as described previously.¹⁰

The concentrations of the following serum and plasma constituents also were determined by previously described methods:¹⁰⁻¹² total protein, total and free cholesterol, phospholipids, esterified fatty acids and protein-bound hexoses, hexosamines, sialic acid, and fucose.

The data were evaluated by the Student *t* test.¹⁴

Results

Myopathy of vitamin E deficiency in rabbits. FIGURE 1 shows the gradual variation of the relative concentrations of electrophoretic fractions of serum proteins and lipoproteins during the development of myopathy in a rabbit. It also includes curves for the ratio of urinary creatine:creatinine and for body

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† Obtained from R. B. Jackson Memorial Laboratory, Bar Harbor, Me.¹³

weight. These two measurements served to follow the progress of the disease.⁴ In the moving-boundary electrophoretic pattern the decrease of albumin and the increase of β globulin were found in every rabbit investigated, and were statistically highly significant ($P < 0.001$). These changes began to develop very early, before the normal rate of weight gain was affected and before the

Development of nutritional muscular dystrophy

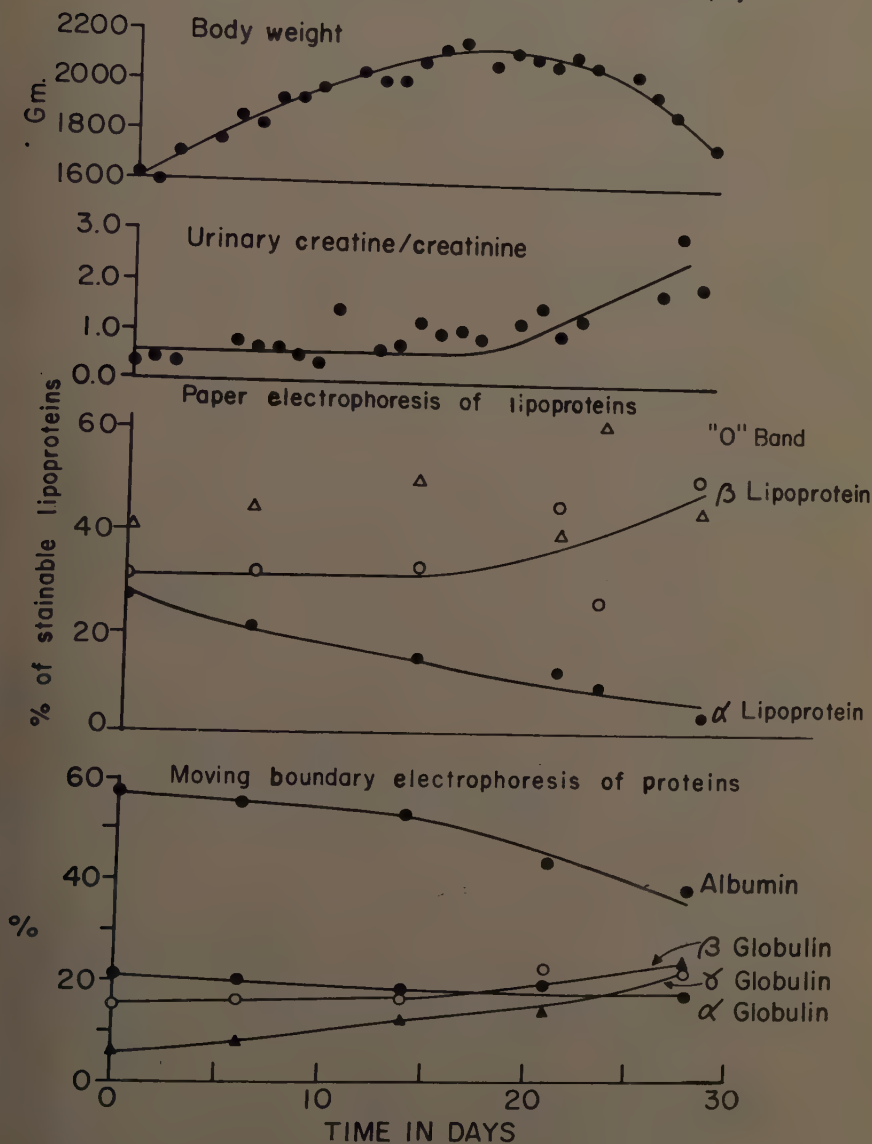


FIGURE 1.

Recovery from nutritional muscular dystrophy

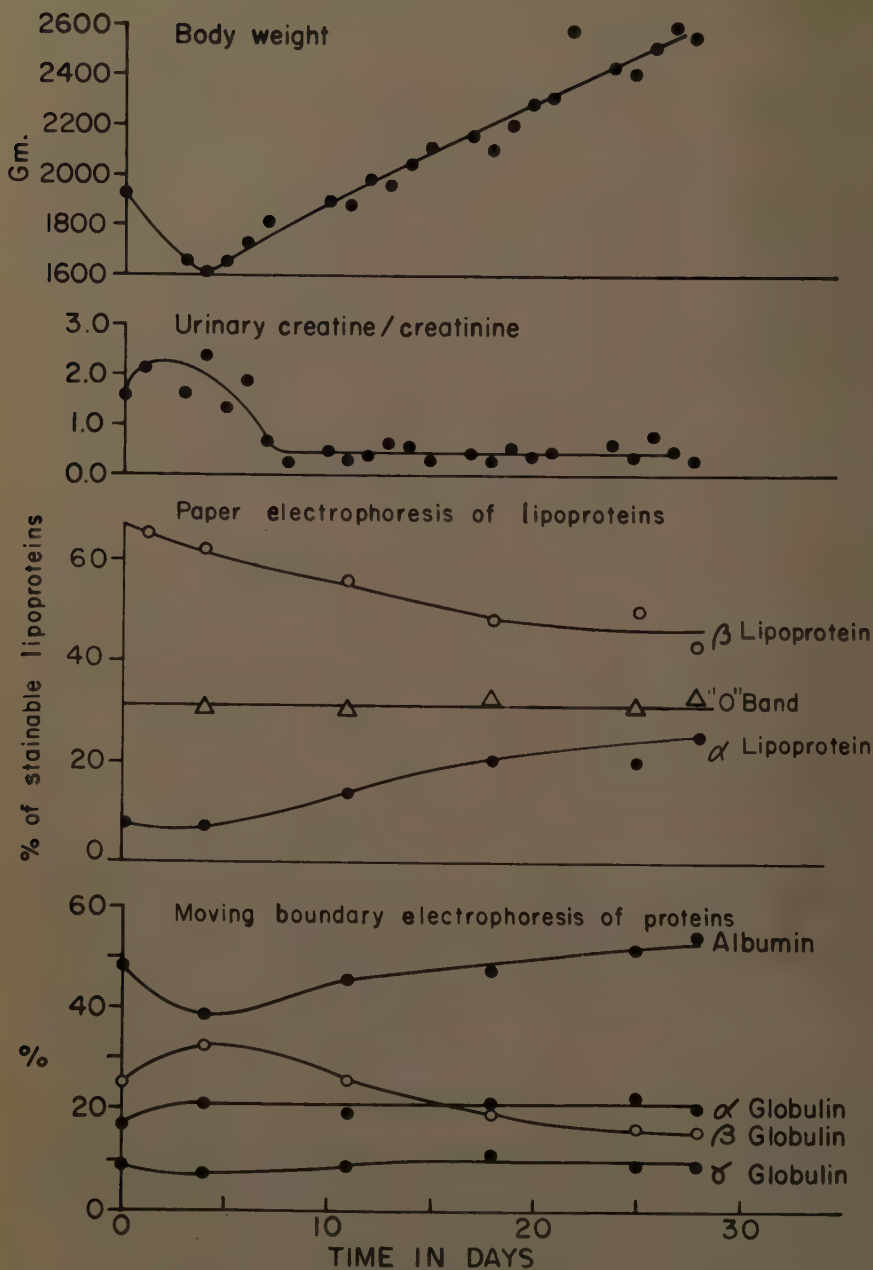


FIGURE 2.

urinary creatine:creatinine ratio had increased appreciably. Changes of the α -globulin fraction, which consisted of 3 partly overlapping peaks, and of γ globulin were not consistent. The mobilities of corresponding serum proteins were alike in dystrophic and normal rabbits. The values for total protein also were not affected by the progress of the disease. The curves for the stainable paper-electrophoretic lipoprotein fractions showed a continuous decrease of α lipoprotein and an increase of β lipoprotein. The values for the "O" band, which trails the β band and is not always sharply separated from the latter, scattered considerably and indicated no definite trend. FIGURE 2 shows the effect of treatment of a vitamin E-deficient dystrophic rabbit with *dl*- α -tocopherol. The electrophoretic patterns of both serum proteins and lipoproteins slowly returned to normal over a period of 4 weeks. They were still improving when the clinical symptoms had already regressed. The relative concentrations of the paper-electrophoretic protein fractions changed with the time in a manner

TABLE 1
SERUM LIPOPROTEINS IN RABBITS DURING DEVELOPMENT OF MUSCULAR
DYSTROPHY AND DURING RECOVERY

Days	Diet	Cholesterol				Phospho- lipids mg. %	Ratio cholesterol/ phospho- lipids
		Total mg. %	Free mg. %	Esteri- fied mg. %	Free/ total %		
7	Vit. E-free						
14	Vit. E-free	126	28	98	22.2	154	0.82
20	Vit. E-free	189	55	134	29.1	208	0.91
21	Vit. E-free + d,l- α -tocopherol						
25	Vit. E-free + d,l- α -tocopherol	297	84	213	28.3	248	1.20
28	Vit. E-free + d,l- α -tocopherol	253	51	202	20.1	250	1.01
32	Vit. E-free + d,l- α -tocopherol	254	58	196	22.8	256	0.99

generally similar to those in the moving boundary patterns of FIGURES 1 and 2. This finding would indicate some changes of the proteins themselves.

TABLE 1 shows that the concentrations of both free and esterified cholesterol and of phospholipids greatly increased during the development of the myopathy and then decreased slowly during recovery. The ratio of free cholesterol to total cholesterol also increased during the progress of the disease but, on treatment, fell quickly to its original value. The ratio of cholesterol to phospholipids was constant during the whole period of progress of the disease but, during recovery, it rose to higher values at first and then approached its final value.

FIGURE 3 shows the distribution of cholesterol and phospholipids among the lipoprotein fractions during the development and subsequent cure of the myopathy in a rabbit. The values have not been corrected for the paper blanks, since the latter fluctuated. The curves varied in a way qualitatively similar to those of the total stainable lipids in FIGURES 1 and 2. The changes of both lipid constituents in the α fraction were more than compensated by their changes in the β fraction so that as a net result their concentrations in whole

serum varied as did those in TABLE 1. The normal values for the ratios of cholesterol to phospholipids for the α and β lipoproteins were about 0.5 and 1.0 respectively, which incidentally were similar to those for normal human serum.¹⁵

DISTRIBUTION OF SERUM CHOLESTEROL AND PHOSPHOLIPIDS AMONG LIPOPROTEINS IN NUTRITIONAL MUSCULAR DYSTROPHY

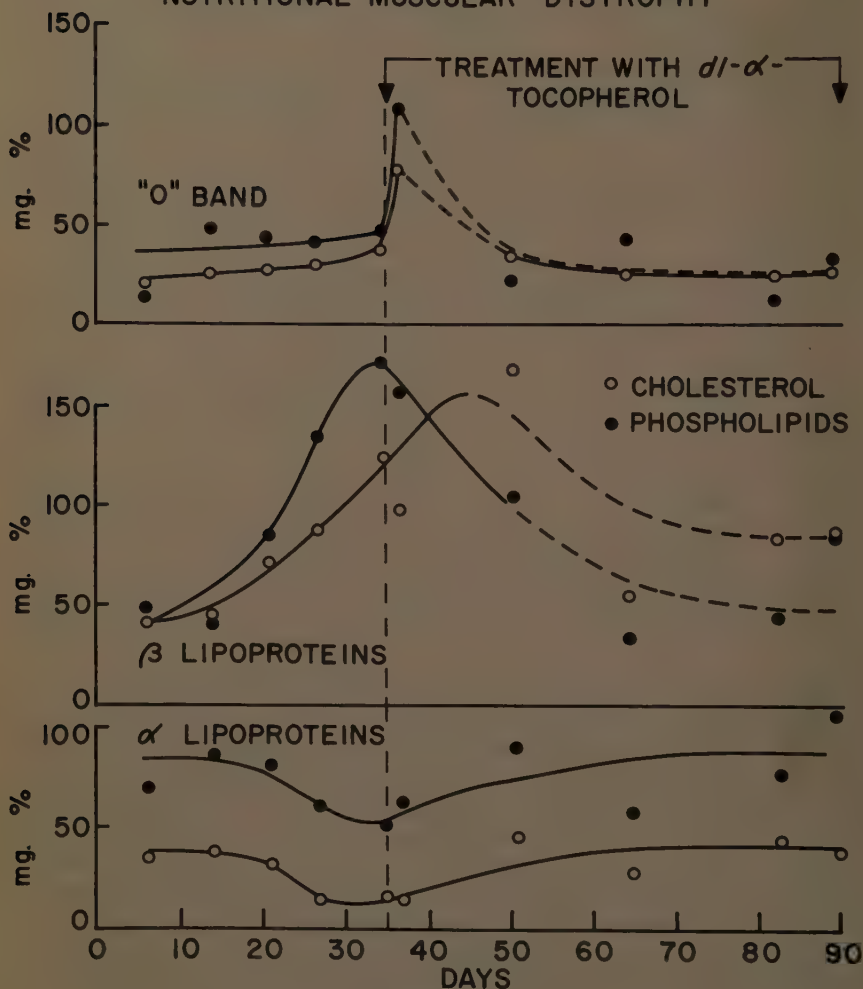


FIGURE 3.

The ratio decreased in both α and β lipoproteins during the progress of the disease. During recovery it soon reached its normal value for the α fraction, but in the β fraction it at first rose to very high values before returning to its normal range.

TABLE 2 indicates that the concentrations of the protein-bound hexoses,

hexosamines, and sialic acid all increased during the development of the myopathy, but to a much smaller extent than did those of the lipids. The concentrations returned to their normal values within 1 to 2 weeks after treatment with *dl*- α -tocopherol had been started. The percentile distribution of the stainable protein-bound carbohydrates among the paper electrophoretic fractions did not seem to vary significantly during the development of the disease.

Muscle diseases in patients. The tables show mean values with standard deviations. Since all patients with PMD, scoliosis, and Perthes' disease were children, they were compared with normal children as controls. However, the patients suffering from myositis, myotonia dystrophica, and amyotrophic lateral sclerosis were adults and, therefore, were compared with healthy adults as controls. The mean values that differed from corresponding ones of their appropriate controls on the statistically significant level of 1 per cent and on

TABLE 2
SERUM GLYCOPROTEINS IN RABBITS DURING DEVELOPMENT OF MUSCULAR
DYSTROPHY AND DURING RECOVERY

Days	Diet	Hexoses mg. %		Hexosamines mg. %	Sialic acid mg. %
		Total	In sero- mucoid fraction		
7	Vit. E-free	75.2	13.0	75.8	65.1
14	Vit. E-free	72.4	12.2	73.4	48.7
20	Vit. E-free	116.5	20.0	91.9	101.0
21	Vit. E-free + <i>dl</i> - α -tocopherol				
25	Vit. E-free + <i>dl</i> - α -tocopherol	93.0	10.4	87.6	80.0
28	Vit. E-free + <i>dl</i> - α -tocopherol	74.9	10.7	82.4	75.4
32	Vit. E-free + <i>dl</i> - α -tocopherol	76.9	11.5	76.9	60.6

the borderline level of 2 per cent are underlined in the tables. Numbers in parentheses are numbers of subjects whenever these differed from those given in the second column of the tables.

TABLE 3 shows the relative concentrations of serum proteins in moving-boundary electrophoretic patterns. Albumin decreased in myositis and scoliosis and α_2 globulin increased in all muscle diseases (the increase in amyotrophic lateral sclerosis, however, could not be shown to be really significant, presumably because of the small number of cases). Other globulins did not vary significantly, with the exception of an increase of γ globulin in scoliosis. The total protein concentration did not change significantly and the electrophoretic mobilities for corresponding fractions were alike in the various groups.

Paper electrophoresis showed the relative concentrations of protein fractions to be alike in patients and controls except for an increase of β globulin in PMD on a 2 per cent level of significance only. In scoliosis, however, albumin was lowered and γ globulin increased, as in moving-boundary electrophoresis; β globulin increased on a 2 per cent level of significance.

TABLE 4 presents the distribution of the stainable lipids among paper-

electrophoretic fractions. Their relative concentrations for the various muscle diseases did not differ much from those of the corresponding controls, but there was a decrease of α lipoprotein in PMD on the 2 per cent level of significance only, and a definite increase of β lipoprotein in myotonia

TABLE 3
MOVING-BOUNDARY ELECTROPHORESIS OF SERUM PROTEINS IN MUSCLE DISEASES

	No. of subjects	Relative concentrations				
		Albumin	α_1 Globulin	α_2 Globulin %	β Globulin	γ Globulin
Pseudohypertrophic M. D.	17	52.7 \pm 2.4	7.0 \pm .8	11.7 \pm 1.1	14.7 \pm 1.8	13.9 \pm 2.2
Myositis	9	46.6 \pm 2.6	7.9 \pm 1.1	12.1 \pm 2.1	15.9 \pm 1.7	17.5 \pm 4.9
Myotonia dystrophica	5	46.3 \pm 10.3	7.1 \pm 1.0	12.5 \pm 1.4	17.4 \pm 3.4	16.7 \pm 10.5
Amyotrophic lateral sclerosis	3	54.9 \pm 4.7	7.0 \pm 1.2	12.0 \pm 2.3	15.9 \pm 1.2	10.2 \pm 7.7
Scoliosis	6	49.9 \pm 1.8	7.2 \pm 1.2	10.8 \pm .9	13.9 \pm 1.0	18.2 \pm 2.4
Perthes' disease	3	52.0 \pm 3.5	6.9 \pm 1.2	11.3 \pm .6	14.1 \pm 2.6	15.7 \pm 3.2
Normal children	8	55.7 \pm 3.0	6.65 \pm .6	10.3 \pm .9	14.7 \pm 1.3	12.65 \pm 3.4
Normal adults	10	53.9 \pm 2.8	7.4 \pm .8	8.9 \pm 1.0	13.9 \pm 1.9	15.9 \pm 1.9

Underlined values, $P < 0.01$.

TABLE 4
PAPER ELECTROPHORESIS OF LIPOPROTEINS IN MUSCLE DISEASES

	No. of subjects	Relative concentrations		
		α l.p. %	β l.p. %	"O" l.p. %
Pseudohypertrophic M. D.	16	35.7 \pm 7.2	44.1 \pm 6.8	20.2 \pm 7.5
Myositis	8	28.2 \pm 6.8	46.8 \pm 10.4	25.0 \pm 11.7
Myotonia dystrophica	3	28.2 \pm 10.2	56.0 \pm 2.5	15.8 \pm 8.7
Amyotrophic lateral sclerosis	3	35.7 \pm 4.7	39.7 \pm 1.7	24.5 \pm 3.9
Scoliosis	6	33.1 \pm 7.3	44.3 \pm 8.4	22.6 \pm 8.9
Perthes' disease	3	30.6 \pm 6.1	44.7 \pm 8.6	24.7 \pm 13.6
Normal children	9	42.3 \pm 5.5	43.8 \pm 6.1	13.9 \pm 5.5
Normal adults	10	33.2 \pm 7.6	43.2 \pm 4.2	23.6 \pm 7.5

Underlined values, $P < 0.01$; broken underlined values, $P < 0.02$.

dystrophica. The distribution of both total phospholipids and sphingomyelins in these lipoproteins was also found to be similar in all groups.

TABLE 5 shows that for whole serum the concentrations of both total cholesterol and phospholipids and also the ratio of the two did not vary significantly; nor did the concentration of free cholesterol. The percentage of free cholesterol in total cholesterol, however, increased in PMD on a 2 per cent level of significance and definitely in scoliosis and Perthes' disease.

TABLE 6 lists the concentrations of protein-bound carbohydrates. Total

hexoses were decreased in PMD, scoliosis, and Perthes' disease, and increased on a 2 per cent level of significance in myositis. The amounts of hexoses in the seromucoid fraction, on the other hand, were similar in all groups and their ratios to total hexoses were fairly constant. The hexosamines varied only

TABLE 5
SERUM CHOLESTEROL AND PHOSPHOLIPIDS IN MUSCLE DISEASES

	No. of subjects	Cholesterol			Phospholipids mg. %	Cholesterol/phospholipids
		Total mg. %	Free mg. %	Free/total %		
Pseudohypertrophic M. D.	17	209 ± 36	51 ± 10	24.6 ± 3.7	232 ± 27	0.91 ± 0.08
Myositis	4	268 ± 101	59 ± 22	22.2 ± 3.8	(15) 267 ± 52	(15) 0.98 ± 0.17
Myotonia dystrophica	3	382 ± 171	92 ± 49	23.8 ± 4.4	358 ± 122	1.05 ± 0.11
Amyotrophic lateral sclerosis	2				227	
Scoliosis	6	195 ± 33	54 ± 8	27.8 ± 3.1	206 ± 35	0.95 ± 0.06
Perthes' disease	3	223 ± 70	60 ± 14	27.4 ± 2.2	281 ± 40	0.78 ± 0.14
Normal children	7	235 ± 20	49 ± 10	20.7 ± 3.1	242 ± 13	0.97 ± 0.07
Normal adults	8	239 ± 28	62 ± 11	25.8 ± 2.3	252 ± 32	0.95 ± 0.05

Underlined values, $P < 0.01$; broken underlined values, $P < 0.02$.

TABLE 6
SERUM PROTEIN-BOUND CARBOHYDRATES IN MUSCLE DISEASES

	Hexoses (mg. %)	Hexosamines (mg. %)	Sialic acid (mg. %)	Fucose (mg. %)
Pseudohypertrophic M. D.	97.6 ± 15.9 (24)	99.5 ± 9.4 (14)	77.5 ± 15.8 (17)	10.1 ± 4.4 (16)
Myositis	126.5 ± 20.2 (5)	110.5 ± 17.1 (5)	84.3 ± 16.7 (5)	12.3 ± 4.0 (3)
Myotonia dystrophica	105.7 ± 22.9 (3)	127.0 ± 12.6 (3)	93.1 ± 17.6 (3)	12.7 ± 6.2 (3)
Scoliosis	82.5 ± 8.9 (6)	107.9 ± 8.4 (5)	65.5 ± 8.5 (6)	10.8 ± .6 (4)
Perthes' disease	75.7 ± 4.6 (3)	92.3 ± 2.3 (3)	69.1 ± 4.0 (3)	8.3 ± 2.6 (3)
Normal children	121.6 ± 11.1 (9)	106.6 ± 5.7 (9)	59.3 ± 8.0 (9)	9.5 ± 1.6 (8)
Normal adults	98.1 ± 9.4 (8)	104.1 ± 10.3 (7)	68.1 ± 10.7 (8)	8.4 ± 2.0 (8)

Underlined values, $P < 0.01$; broken underlined values, $P < 0.02$.

in Perthes' disease in which they were decreased. Sialic acid was the only protein-bound carbohydrate that increased significantly in PMD. Its apparent elevation in other muscle diseases was not of statistical significance because of large deviations and the small numbers of subjects. Fucose, a minor constituent, followed the same trend. The percentile distribution of stainable carbohydrates among paper-electrophoretic fractions was alike in all groups.

Hereditary muscular dystrophy in mice. The relative concentrations of serum proteins in paper-electrophoretic fractions from dystrophic mice did not

differ significantly from those from normal littermates. The paper-electrophoretic pattern for lipoproteins consisted of an intense band at the location of albumin (occasionally extending somewhat ahead of the latter) and of a band spread widely among the globulins. The relative concentration of the former band considerably decreased from 59.3 ± 9.3 per cent in normal mice to 42.7 ± 8.6 per cent in dystrophic mice, which was on the 0.1 per cent level of very high significance. The other band increased correspondingly, but its diffuseness did not permit any definite differentiation among the various globulins. The pattern of stainable glycoproteins consisted of 3 bands at the locations of α_1 , α_2 , and β globulins. The band associated with α_2 globulin was significantly lowered.

TABLE 7 shows that the concentrations, in plasma, of protein nitrogen and of protein-bound lipids and carbohydrates were somewhat lower, but not significantly so in dystrophic mice than in controls.

TABLE 7
PLASMA PROTEINS AND PROTEIN-BOUND LIPIDS AND CARBOHYDRATES OF MICE
WITH HEREDITARY MUSCULAR DYSTROPHY

	Dystrophic mice	Control littermates
Protein nitrogen, gm. %	0.828 ± 0.092	0.838 ± 0.096
Total cholesterol, mg. %	131 ± 33	132 ± 34
Esterified cholesterol, mg. %	89 ± 24	83 ± 24
Free cholesterol, mg. %	42 ± 18	49 ± 26
Free chol./total chol. %	32.0 ± 10.8	36.0 ± 13.4
Phospholipids, mg. %	188 ± 32	204 ± 50
Cholesterol/phospholipids	0.67	0.65
Esterified fatty acids, mg. %	237 ± 114	398 ± 253
Hexoses, mg. %	81.1 ± 31.1	84.8 ± 17.5
Hexosamines, mg. %	75.8 ± 15.1	86.4 ± 20.0
Sialic acid, mg. %	110.1 ± 24.2	128.4 ± 26.7

The group of normal littermates used as controls consisted of both heterozygous and homozygous individuals, depending on the type of mating. To exclude the possibility that the heterozygous littermates were affected by mutation, the same series of determinations was performed with a group of normal homozygous mice. The values for the two control groups did not differ significantly except for a doubtful decrease in the ratio of free to total cholesterol in the homozygous group.

Discussion

The significant changes in the concentrations of albumin and β globulin in the electrophoretic patterns for the myopathy of vitamin E deficiency in rabbits represented, to some extent, slow and moderate changes of the proteins themselves. The increase of β globulin, however, was related primarily to the strikingly elevated levels of cholesterol (both free and esterified) and phospholipids. The marked shift in the paper-electrophoretic pattern of the lipoproteins also indicates that this myopathy is characterized by a severe disturbance of the lipid metabolism. The protein-bound carbohydrates did

not increase nearly as much during the progress of the disease and, on treatment, they returned to their normal values more rapidly.

In contrast to this nutritional form of muscular dystrophy our studies of muscle diseases in patients generally did not indicate any pronounced changes in the lipid constituents. However, Dryer *et al.*¹⁶ found the level of phospholipids in PMD to be decreased to less than one-half normal value and observed some other changes in the lipoproteins and proteolipids. Danowski *et al.*¹⁷ reported a reduction in total cholesterol, but we found such a decrease only on a 5 per cent level of significance.

Various changes of the electrophoretic patterns of serum proteins in patients with muscle diseases have been reported.^{16,18-26} These findings often showed discrepancies in the results. The increase of α_2 globulin, however, has been confirmed several times.^{16,19,25,26} Dryer *et al.*¹⁶ described a twinning of the α_2 -globulin peak as a nearly regular and characteristic feature of PMD, but we observed this only occasionally. The peak was usually asymmetric and broad, indicating electrophoretic heterogeneity. Jackson and Block¹⁹ found an abnormal resolution of α_2 globulin in patterns of patients with a recessively inherited muscular dystrophy, presumably PMD and, interestingly enough, also in those of the parents of these patients. The increase of α_2 globulin resulted from an increase of conjugated nonprotein constituents rather than of proteins themselves, since the latter did not change in paper-electrophoretic patterns. It probably was related to the increase of sialic acid that has high affinity for α_2 globulin.^{27,28} It was not specific for muscle diseases, and has been found in other wasting diseases.²⁹

Diseases with tissue destruction are usually accompanied by high levels of sialic acid and of the two other main protein-bound carbohydrates, hexoses, and hexosamines.²⁹ In muscle diseases in patients, however, the two latter constituents did not increase significantly. Since the value for hexoses in normal adults was lower than the range reported in the literature,²⁹ the moderate increase of this substance in myositis may be only apparent. In PMD the hexoses were actually decreased, which may be related to their reported low value in cerebrospinal fluid.³⁰ In the nutritional myopathy, on the other hand, the levels of all the main protein-bound carbohydrates were elevated.

In the hereditary muscular dystrophy in mice the only significant changes found were shifts in the distribution of lipoproteins and, to a lesser extent, of glycoproteins. The concentrations in whole serum of cholesterol³¹ and phospholipids and of the protein-bound carbohydrates did not increase. In this respect the myopathy in mice resembled the human muscle disease in which hereditary factors also play an important role. On the other hand the lipoprotein patterns were greatly altered both in the hereditary myopathy in mice and in the nutritional muscular dystrophy in rabbits. Therefore it is not clear whether the morbid process in mice resembles the hereditary muscle diseases in man more closely than the state of deficiency in rabbits. Since these three forms of muscular dystrophy involve three different species, only cautious comparisons should be made.

It is noteworthy that the serum protein concentration remained unchanged in each of the three kinds of muscular dystrophy, in spite of the pronounced loss of muscle proteins.

Summary

In the myopathy of vitamin E deficiency in rabbits the electrophoretic pattern revealed a decrease of albumin and an increase of β globulin already during the very early stages of the disease. The increase of β globulin was associated with large amounts of both free and esterified cholesterol and of phospholipids. The magnitude of these changes and the slow return to normal values with administration of vitamin E indicated a severe disturbance of the lipid metabolism. The protein-bound carbohydrates (hexoses, hexosamines and sialic acid) increased only moderately and, on treatment, soon returned to their normal levels.

Various forms of muscle disease in patients were investigated: pseudohypertrophic muscular dystrophy (PMD) in particular, myositis, myotonia dystrophica and amyotrophic lateral sclerosis, and scoliosis and Perthes' disease with occasional secondary muscular changes. They did not show the extensive alterations, particularly of the lipoproteins, which were characteristic of the nutritional muscular dystrophy in rabbits. The only definite change in nearly all the muscle diseases was a significant although not specific increase of α_2 globulin in the moving-boundary electrophoretic patterns. This increase seemed to be related to a high level of sialic acid, particularly in PMD. The other two main protein-bound carbohydrates did not show the significant increase that was observed with the myopathy of vitamin E deficiency in rabbits and that often is associated with other wasting diseases.

In the hereditary muscular dystrophy in mice the concentrations of these constituents did not differ from those in controls. In this regard the myopathy in mice resembled the hereditary muscle diseases in man, but it differed from them by a shift in the distribution particularly of lipoproteins and, to a smaller extent, of glycoproteins among the electrophoretic fractions.

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LIPOPROTEINS AND THEIR RELATION TO METABOLIC DISEASE*

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The broad subject *Lipoproteins and Their Relation to Metabolic Disease* offers an opportunity to present some of the results of studies conducted for the last 10 years on several groups of patients with various metabolic disorders, including normal persons and specially selected ethnic groups.

Serum lipoproteins were studied ultracentrifugally at a density of 1.21, using NaCl-KBr.¹ To simplify the terminology in presenting the results, the following scheme, as suggested by Furman (personal communication) with slight modifications, is used: the fractions in order of decreasing flotation rate at density of 1.21 are designated V.F.R., a very fast rising fraction that includes most of the chylomicrons, and has a flotation rate $-S > 400$; V.L.D., fractions with flotation rates between $-S 70-400$ and approximately equivalent to S_f 20-100; L.D. β , $-S 40-70$, equivalent to S_f 12-20; β , $-S 25-40$ equivalent to S_f 3-12; α_2 , $-S 20-25$, equivalent to S_f 0-3; and α , $-S 1-10$, not floated at density 1.063 (TABLE 1).

Estimation of total serum cholesterol was made by the method of Abell *et al.*² All blood was drawn when the subjects were in the postabsorptive state.

GENETIC FACTOR

Genetic, nutritional, hormonal, and hepatic factors are known to be important in determining a person's normal serum lipoprotein pattern. The importance of the genetic factor is evident when the patterns of normal white Clevelanders and of Navajo and Peruvian Indians are compared. The serum cholesterol concentrations of normal Navajo and Peruvian Indian men³ are lower than that of normal white men of the Cleveland, Ohio, area, and a relatively greater proportion of the serum lipoproteins of the Indians occurs as α , than as β plus low-density fractions, than in the serum of Cleveland white men, the ratios being 0.56 and 0.46 respectively (TABLE 2). Since the normal concentration of serum cholesterol of the Indians is lower than that of the Cleveland men, comparison of the Indians' lipoprotein pattern was made with that of Trappist monks, who also have a relatively low normal concentration of serum cholesterol.⁴ There was a similar but somewhat greater difference in the ratios of $\alpha:(\beta + \text{low-density fractions})$ between the Indians and the monks, 0.56 and 0.42 respectively, than between the Indians and the Clevelanders.

The role of the genetic factor in determining the serum lipoprotein pattern is also evident in patients with familial hyperlipemia, more correctly termed hyperglyceridemia, in whom the concentrations of V.F.R. and V.L.D. lipo-

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protein fractions of serum are greatly increased, while the concentrations of β lipoprotein are decreased, and those of α lipoprotein are in the low normal range or are decreased. In contrast, the serum of persons with hereditary hypercholesterolemia have an increased concentration of L.D. β and β lipoproteins with normal amounts of lower density fractions, while the changes in α lipoprotein are variable. In both types of hereditary abnormalities in lipid metabolism, the ratios of $\alpha:(\beta + \text{low-density fractions})$ are lower than those of either normal white, or normal Indian subjects (TABLE 2). Coronary disease is rare among the Indians,⁵ whereas in persons with hereditary defects in

TABLE 1
CLASSIFICATION OF SERUM LIPOPROTEINS SEPARATED BY
ULTRACENTRIFUGATION OR ELECTROPHORESIS

Abbreviation	Serum lipoprotein fraction					
	V.F.R.	V.L.D.	L.D.	β -	α_2	α_1
Ultracentrifugation density 1.21, -S	>400	70-400	40-70	25-40	20-25	1-10
density 1.063, S _f	>100	20-100	12-20	3-12	1-3	Not flo- tated
Electrophoresis	Remains at origin		β -	β -	α_2	α_1

TABLE 2
RELATIVE PROPORTION OF α AND β LIPOPROTEINS IN SERUM OF NAVAJO AND PERUVIAN
INDIANS,⁴ OF WHITE MALES OF THE CLEVELAND AREA, OF TRAPPIST MONKS,⁸ AND OF
PATIENTS WITH HEREDITARY ERRORS IN LIPID METABOLISM

Group	Serum cholesterol mg./100 ml.	$[\alpha \text{ lipoprotein } (-S \text{ 1-10})]/$ $[\beta + \text{low-density lipoproteins}$ $(-S \text{ 25-400})]$
Navajo Indians	175 \pm 10	0.56
Peruvian Indians	186 \pm 11.8	0.56
Cleveland white males	232 \pm 8	0.46
Trappist monks	209 \pm 11	0.42
Hereditary hyperlipemia	422	0.13
Hereditary hypercholesterolemia	376	0.20

lipid metabolism it is even more common than in normal white subjects. Heredity plays an important part in determining the serum lipoprotein pattern and, in turn, greater incidence of atherosclerosis is found in persons having a relatively large proportion of serum lipoproteins as β , and low-density lipoprotein fractions. The answer of a 92-year-old physician when asked: "What is your secret for longevity?" was, "I chose my parents wisely." This answer may well have a sound scientific basis.

NUTRITIONAL FACTOR

The importance of diet in determining the serum lipoprotein pattern has been extensively studied. Previous studies have shown the benefit of low-fat diets, or diets rich in unsaturated fat, on the serum cholesterol concentration

and the lipoprotein pattern;⁶ however, most of the subjects have been followed for relatively short periods, and some of the diets are of the formula type. A program* has been in progress for the past four years at the Cleveland Clinic to determine the effects of diets adhered to for long periods on the serum cholesterol concentrations and lipoprotein patterns of patients with elevated blood lipids, and to develop a palatable, acceptable diet that will produce beneficial changes in serum lipid concentration.

According to our studies, the serum lipoprotein patterns of patients with high serum cholesterol concentrations may show one of three types of changes:

(1) Hyperglyceridemia, characterized by lipoprotein concentrations of V.F.R. and V.L.D. fractions several hundred per cent greater than normal, and a β - and α -lipoprotein concentration in the low normal range or lower (TABLE 3). This type of pattern is associated with extremely high concentration of serum triglycerides.

(2) Hypercholesterolemia, characterized by L.D. β and β -lipoprotein concentration 100 to 500 per cent greater than normal with no, or inconstant, changes

TABLE 3
THREE TYPES OF LIPOPROTEIN PATTERNS OBSERVED IN PATIENTS
WITH DIFFERENT TYPES OF HYPERLIPEMIA

Type of lipoprotein abnormality	Cholesterol	-S > 400	70-400	40-70	25-40	20-25	1-10
		mg./100 ml. serum					
Hyperglyceridemia	422	++++*	737	142	174	35	145
Hypercholesterolemia	376	0	81	126	726	28	171
Mixed hyperlipemia	323	++*	164	130	474	24	148

* Cannot be quantitated, but amount estimated as 0 to +++++.

in the other fractions. A high concentration of serum cholesterol, with no increase in triglycerides is associated with this type of pattern.

(3) Mixed hyperlipemia, our term for a change characterized by a high concentration of all low-density and β -lipoprotein fractions and variable changes in α -lipoprotein concentration. High cholesterol, triglyceride, and phospholipid concentrations, with all in essentially normal proportions, are associated with this type of pattern (TABLE 3).

A low-fat diet usually has been given to the patients after completing the control studies. This food pattern, which provides approximately 12.5 per cent of the calories as fat and 14 per cent as protein has been continued for from 3 to 4 months, when a change is made to a "vegetable-oil" pattern. This diet provides approximately 40 per cent of the calories as fat, of which 5 per cent is meat fat, 30 per cent cotton seed oil, and 5 per cent "basic" fat (from cereals, vegetables, and fruits), and 14 per cent protein. Some of the subjects have followed this diet for more than 3 years.

Patients with hyperglyceridemia who adhered well to the diet, whether of a

* Carried out under the direction of I. H. Page with the collaboration of Helen B. Brown, Harriet P. Dustan, and Lena A. Lewis.

low-fat or vegetable-oil pattern, have shown decreased concentrations of serum cholesterol and V.F.R. and V.L.D. lipoproteins. The changes in lipoprotein concentrations observed in one of the 10 patients with hyperglyceridemia are shown in FIGURE 1 and are typical of the results obtained when the diets are followed for relatively long periods. Although the concentrations of the low-density fractions decreased, the typical pattern occurring in hyperglyceridemia was still evident, as the concentration of β and α lipoproteins remained low. The variation of cholesterol concentrations from time to time was less, when the subjects were on the vegetable oil food pattern than when they were on the low-fat regimen. Any variation in body weight of the subjects was reflected by a corresponding increase or decrease in concentration of blood lipids. There is no indication that the amounts of serum cholesterol and lipoproteins

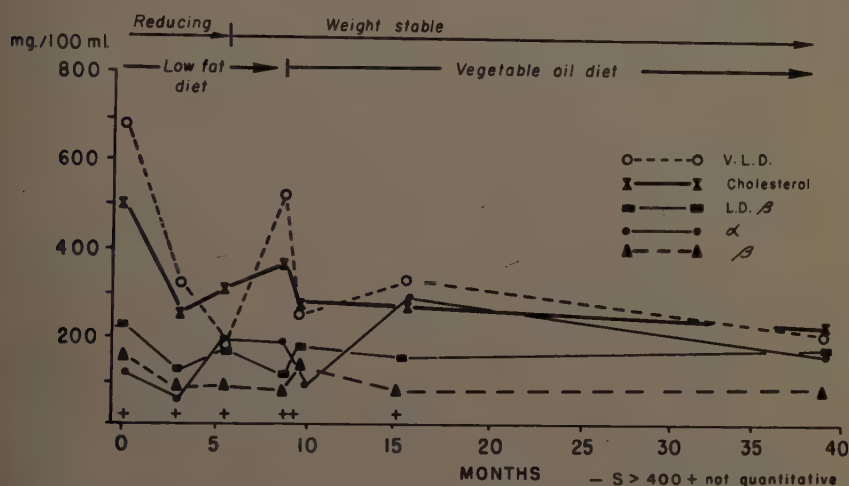


FIGURE 1. The serum lipoprotein and cholesterol concentrations of a patient with hyperglyceridemia before and when adhering to special food patterns.

tend to revert to pretreatment values within a period of three years when the patients are eating, according to the special food patterns.

The 19 patients with hypercholesteremia when on the diet program showed much more variable changes than the 10 patients with hyperlipemia. In the 19 patients, serum cholesterol content decreased by from 7 to 40 per cent, the extent of decrease showing no relation to the pretreatment serum cholesterol concentration. The decrease in serum cholesterol concentration was usually associated with a proportional decrease in concentration of β and L.D. β lipoproteins, while the α lipoprotein was either unchanged or, if initially very low, sometimes increased. After a period of months, even with careful dietary adherence, the serum cholesterol and lipoprotein pattern often tended to revert toward pretreatment levels. The serum cholesterol and lipoprotein patterns in hypercholesteremia did not reflect a change in body weight of the subjects as they did in hyperglyceridemia.

We were fortunate in having an opportunity to study changes in the lipo-

protein pattern that occur during pregnancy in two hypercholesteremic patients who had been on dietary management for several years. Comparisons were made of prepregnancy, pregnancy, and postpartum serum-lipoprotein patterns. The subjects adhered faithfully to the vegetable-oil food pattern during the entire period of study. The changes in the serum lipoprotein pattern in hypercholesteremia were similar to those reported by Oliver and Boyd⁷ for normals, except that the initial levels were significantly higher than in the normal. The serum cholesterol concentration increased and the relative pro-

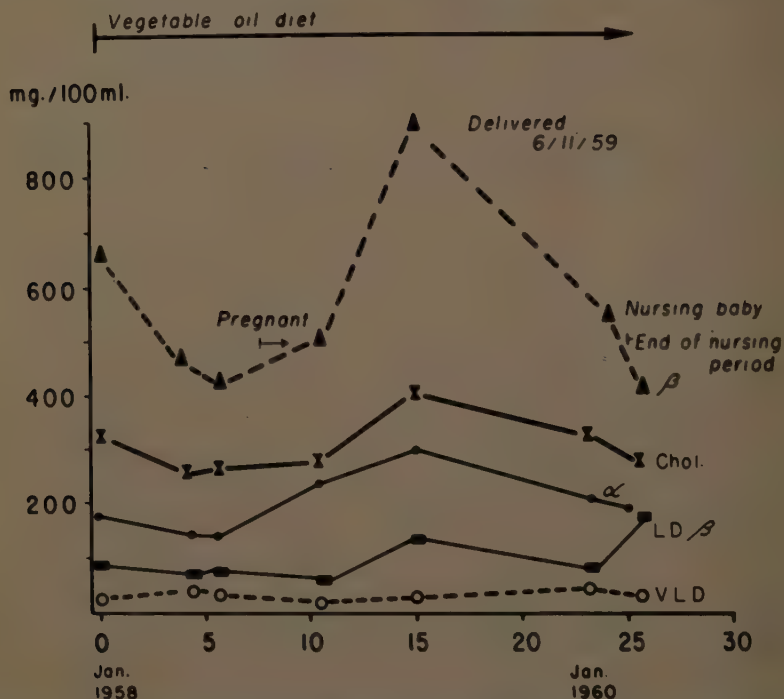


FIGURE 2. Changes in the serum lipoprotein pattern of a patient with hypercholesteremia before, during, and following pregnancy, and adhering to the "vegetable-oil food pattern."

portion of β -lipoprotein was augmented. In the postpartum state after termination of lactation the levels returned to prepregnancy values (FIGURE 2).

Treatment of the patients with mixed hyperlipemia by dietary management resulted in a gradual return of the serum lipoprotein pattern to normal levels. Four of the five patients observed for a year or longer had normal values. The pattern did not revert to that of the pretreatment period, and dietary management was entirely effective in maintaining a normal lipoprotein distribution (FIGURE 3).

The serum-lipoprotein pattern can be benefited greatly by dietary treatment if the pattern is of the hyperglyceridemia or the mixed hyperlipemia type. The pattern in patients with hypercholesteremia is less consistently influ-

enced by diet, and the change is not as well maintained over long periods as it is in patients with the other types of abnormal lipid patterns.

HORMONAL FACTORS

Thyroid Hormone

Many types of hormonal imbalance result in changes in serum lipoproteins. An increase in the serum-cholesterol concentration in many patients with hypo-

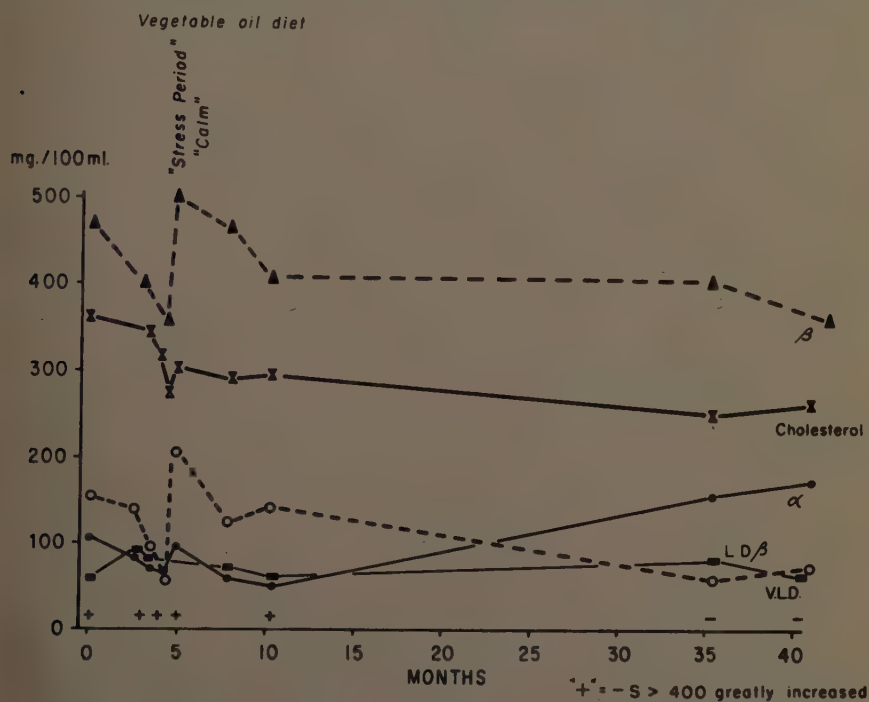


FIGURE 3. The serum lipoprotein and cholesterol concentrations of a patient with "hyperlipemia, mixed-type, before and when adhering to special food patterns.

thyroidism and a decrease in hyperthyroidism was reported in 1922 by Epstein and Lande.⁸ Gofman *et al.*⁹ reported an increased concentration of S_f 0-12 lipoproteins in 2 patients with myxedema, and Jones *et al.*¹⁰ observed increased concentrations of all lipoprotein fractions, when determined at density 1.21, in serum of hypothyroid patients. Studies by Gould and his associates¹¹ have shown that the increased cholesterol and lipoprotein concentration in hypothyroidism is associated with a decreased rate of synthesis and a proportionately greater decrease in the rate of catabolism of cholesterol and lipoproteins.

In our studies no difference was observed between the lipoprotein pattern of patients with spontaneously occurring myxedema and that developing after treatment with radioiodine (I^{131}), as shown in TABLE 4. In both groups changes of 2 types were observed.

TABLE 4
SERUM LIPOPROTEIN PATTERN OF MYXEDEMATOUS AND HYPERTHYROID PATIENTS BEFORE AND FOLLOWING TREATMENT

	Patient initial and sex	Age (years)	Evaluation or treatment	B.M.R.	Cholesterol	Lipoproteins (mg./100 ml. serum)				
						-S70-400	40-70	25-40	20-25	1-10
Myxedema A. Following thyroidectomy	S ♀	64	Hypothyroid	-24	620	40	118	>823	24	270
	M ♀	35	No evidence of hypothyroidism. 2 gr. thyroid per day for 2 yr. Diabetes and hypothyroidism TSH (10 USP units per day for 18 days)	-28	368	32	80	510	20	230
	C ♂	47	Thyroid gr. 2/day 6 weeks	-6	231	16	33	215	19	275
	A ♀	38	Hypothyroid 2 gr. thyroid/day for 6 weeks	-32	283	160	104	424	28	235
B. Spontaneous	K ♀	48	Hypothyroid 2 gr. thyroid/day, 4 weeks	-4	174	10	28	285	20	275
	R ♀	33	Hypothyroid 2 gr. thyroid/day, 4 weeks	-20	380	39	117	600	5	260
	L ♂	46	Hypothyroid 2 gr. thyroid/day, 4 weeks	-10	192	59	36	270	14	201
	W ♂	47	Hypothyroid 2 gr. thyroid/day, 3 months	-23	501	35	141	>550	24	322
Hyperthyroidism	S ♀	57	Hypothyroid 2 gr. thyroid/day, 6 months	-26	389	88	104	306	14	136
	R ♀	29	Hypothyroid 2 gr. thyroid/day, 6 months	+4	149	30	38	152	9	141
	S ♀	57	Hypothyroid 2 gr. thyroid/day, 6 months	-30	195	9	42	228	7	175
	R ♀	29	Hypothyroid 2 gr. thyroid/day, 6 months	-11	362	124	90	>300	28	168
			Hyperthyroid, "Graves"	+41	200	48	52	290	22	190
			2 months following 16 m.c. I ¹³¹	-14	151	66	28	199	16	92
			Hyperthyroid, "Graves"	+56	245	80	35	300	16	140
			2 years after 12 m.c. I ¹³¹	+9	131	5	16	143	16	212
			Hyperthyroid, "Graves"	+66	210	10	10	270	10	270
			3 months after 12 m.c. I ¹³¹	-12	162	7	7	129	19	188
					225	14	24	260	12	248

(1) A 100 to more than 500 per cent increase in concentration of β and low-density lipoprotein fractions and from 20 to 100 per cent increase in α -lipoprotein concentration (patients S, M, A, K). This type of change had been reported by Jones *et al.*¹⁰

(2) Greatly increased concentrations of V.L.D. and L.D. β and proportionately smaller increases in β -lipoprotein; α -lipoprotein concentrations were low (patients C, R, L). After treatment with thyroid hormone, in patients having the first type of pattern, the concentrations of all serum lipoproteins decreased, while in patients with the second type of pattern there occurred a decrease in concentrations of all classes of low-density and β -lipoproteins and increase in concentration of α -lipoprotein. These studies suggest a possible primary failure of α -lipoprotein synthesis in hypothyroid patients with the second type of lipoprotein pattern, a failure that is corrected by treatment with thyroid hormone. In hyperthyroidism the concentrations of serum cholesterol and lipoproteins are decreased and, after treatment, when the patients had a normal metabolic rate, the concentrations of all serum lipoprotein fractions and cholesterol increased.

The efficacy of thyroid hormone therapy in reducing the concentrations of serum lipoproteins of hypothyroid patients from the greatly elevated levels characteristic of the disease, has stimulated extensive investigation by several groups, of the effects of various thyroid compounds on lipoproteins and lipids of euthyroid persons with high blood lipid content, in an endeavor to find a thyroid analogue that has greater effect on lipid metabolism than on metabolic rate.^{12,13} The results are sufficiently promising to justify further intensive study.

Pancreatic Hormones

Insulin. Since vascular disease develops more frequently in diabetics than in the population as a whole, it was hoped that a study of the serum lipoproteins in diabetes would give information to explain this difference. Whereas uncontrolled diabetics with acidosis have a high concentration of V.F.R. and V.L.D. lipoproteins and, frequently, increased concentrations of L.D. β and β -lipoproteins, in controlled diabetics the V.F.R. and V.L.D. fractions quickly decrease to nearly normal amounts. Hanig and Lauffer¹⁴ found no clear difference in concentrations of S_f 12-20 lipoproteins among diabetic males (with or without cardiovascular complications) and normal males, when both high and low values of S_f 12-20 were given equal weight in the analysis. In more detailed classification of the diabetic state others^{15,16} observed a greater incidence of high S_f 12-20 values in "fairly well" and poorly controlled diabetics than in well controlled ones, but the differences were not so great as might have been expected. Studies of Albrink and Man¹⁷ showed a rapid shift in the concentration of serum triglyceride in some diabetics when they were on their usual program of insulin and diet, which suggests that lipid changes in diabetics may be very transient. This may partially explain the failure to detect great differences in lipoprotein concentrations, since studies are usually made when subjects are postabsorptive in the morning.

Lipoproteins were studied in a "stable" and in a "brittle" group of diabetics;

the classification of type of diabetes was made by the criteria outlined by Ali-visatos and McCullagh.¹⁸ All of the subjects had diabetes for at least 10 years, were more than 35 years of age, and the diabetes was in good control at the time of study. The serum cholesterol concentrations of stable and brittle diabetics of both the middle-aged and old-age groups were similar. As in normal subjects,¹⁹ the cholesterol concentrations of the diabetic women continued to increase with age, while those of middle-aged and old, diabetic men were similar. The range of concentration of the various lipoproteins within groups was great. TABLE 5 summarizes the results, which show a higher mean concentration of L.D. β and β -lipoprotein both in stable and in brittle diabetics than in normals. The concentration of L.D. β was somewhat higher in

TABLE 5
LIPOPROTEIN PATTERN OF MIDDLE-AGED AND OLD PATIENTS
WITH STABLE OR BRITTLE DIABETES

	(mg./100 ml.)						Age (years)	Known duration of diabetes (years)
	-S70-400	40-70	25-40	20-25	1-10	Choles- terol		
Women								
Middle-aged (35-60)								
Diabetes, stable (5)	45	92	527	24	209	262	51	12
Diabetes, brittle (19)	30	49	400	23	296	266	45	14
Old (61-80)								
Diabetes, stable (18)	29	60	400	25	239	276	70	17
Diabetes, brittle (3)	16	44	400	18	232	281	73	26
Normal (35-60)	25 \pm 4	32 \pm 4	296 \pm 16	16 \pm 2	244 \pm 14	242 \pm 10	42	
Men								
Middle-aged (35-60)								
Diabetes, stable (6)	40	79	455	27	216	255	45	16
Diabetes, brittle (6)	55	63	427	33	289	249	50	13
Old (61-80)								
Diabetes, stable (15)	26	49	400	20	179	242	69	16
Diabetes, brittle (5)	37	66	400	31	183	252	66	15
Normal (35-60)	60 \pm 10	33 \pm 1	285 \pm 17	14 \pm 1	180 \pm 5	232 \pm 8	43	

stable than in brittle middle-aged diabetics. The V.L.D. lipoprotein concentration of all groups was normal as was the α -lipoprotein in both old stable and brittle diabetics. The α -lipoprotein concentrations of middle-aged brittle diabetic men and women was high. In general, the number of middle-aged brittle diabetics having vascular complications is somewhat less than of stable diabetics of the same age.

Well-controlled diabetics on a constant diet have a relatively constant lipoprotein pattern over a period of years. Similarly, small changes have been observed in normal subjects.²⁰ TABLE 6 summarizes studies extending over three or more years on well-controlled diabetics. Those having high lipoprotein and serum cholesterol, or low levels showed equally constant values. Rapid changes in concentration, however, may occur if the diabetes goes out of control. In general, the lipoprotein concentration showed little correlation with the incidence of vascular disease, unless there was renal involvement.

The lipoprotein pattern of the 10 patients with intercapillary glomerulosclerosis showed a high concentration of V.L.D. and L.D. β -lipoproteins (TABLE 7). The β -lipoprotein concentration was increased in 7, normal in 2 and low in 1, while that of the α -lipoprotein was decreased in 8 and normal in 2 subjects. The patients, who ranged in age from 22 to 43 years, had had diabetes for from 8 to 17 years. In intercapillary glomerulosclerosis it appears that the balance of triglyceride uptake and release (indicated by high concen-

TABLE 6
SERIAL SERUM LIPOPROTEIN STUDIES ON PATIENTS WITH
WELL-CONTROLLED DIABETES

Patient, initials, sex	Date	mg./100 ml. serum					
		-S70-400	40-70	25-40	20-25	1-10	Choles- terol
M.A. ♀	7-24-1951	151	156	391	10	348	270
	10-3-1957	35	157	425	26	375	264
D.C. ♀	1-22-1954	16	190	>1000	59	270	602
	10-1-1956	24	194	>1100	24	358	604
M.W. ♀	1-29-1954	59	94	>390	10	259	317
	12-1-1956	47	84	496	14	132	290
M.V. ♂	9-10-1954	42	35	>300	21	180	267
	1-25-1957	47	87	>350	24	248	269
S.N. ♀	2-18-1954	12	26	>152	12	194	158
	7-2-1956	11	9	223	7	229	184
W.M. ♂	2-18-1953	21	63	456	21	212	192
	8-16-1956	33	52	359	19	250	189
J.S. ♂	3-15-1955	42	168	882	27	318	337
	4-11-1958	23	90	600	16	285	298

TABLE 7
SERUM LIPOPROTEIN PATTERN OF PATIENTS WITH INTERCAPILLARY GLOMERULOSCLEROSIS

Patient, initials, sex	Age (years)	Dura- tion of diabetes (years)	Clinical notes	mg./100 ml. serum					
				-S70- 400	40-70	25-40	20-25	1-10	Choles- terol
R.D. ♂	25	13	Diabetes well-controlled; arteriosclerosis severe	280	94	610	82	136	377
P.D. ♂	43	8	Diabetes, good control	190	40	294	16	152	210
R.F. ♀	33	17	Diabetes, good control; early i.g.	104	71	600	12	54	326
J.G. ♂	31	16	Retinopathy IV; kidneys also involved	118	212	>400	19	143	406
E.P. ♀	38	?	Brittle diabetic	94	214	>1000		141	562
J.S. ♂	35	16	Angina pectoris	90	113	333	19	178	232
R.U. ♂	37	15	Poor control; labile, early i.g.	157	73	480	14	87	260
C.S. ♂	32	10	Well controlled	82	98	350	16	122	205
M.S. ♀	22	8	I.g. associated with chronic pyelonephritis	46	68	104	10	250	163
F.D. ♀	35	13	Fair control	848	666	472	132	116	353

tration of V.L.D. lipoproteins) is not adequately adjusted, even though the diabetes, as judged by blood glucose levels is well controlled.

In a more complete characterization of the blood lipids in diabetics, Hallgren and his co-workers²¹ found that the fatty acid composition, determined by gas chromatography, of serum lipids of diabetics with vascular complications was similar to that of normal individuals. Thus, while abnormal serum lipoprotein concentrations may be present in some diabetics, the fatty acid composition of the lipoproteins is normal.

Glucagon. While insulin is known to be important to the regulation of lipid metabolism, any effect of glucagon, the hyperglycemic factor of the pancreas, is not well documented. Oliver and Boyd²² noted only equivocal changes in serum cholesterol concentration after infusion of glucagon into human beings. Our studies on diabetic and nondiabetic subjects showed a small, transient but significant decrease in serum cholesterol concentration following infusion of glucagon (TABLE 8). The degree of significance was greater in the diabetic than the normal subjects, and was greatest 1 hour after administration of

TABLE 8
CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF HUMAN
BEINGS FOLLOWING INJECTION OF GLUCAGON

	Preinjection cholesterol mean \pm S.D.	After injection of glucagon (minutes)		
		30 d \pm S.E.*	60 d \pm S.E.*	120 d \pm S.E.*
Diabetic	239 \pm 38.0	7.7 \pm 2.72†	9.0 \pm 3.56†	6.9 \pm 3.50
Nondiabetic	239 \pm 24.8	11.7 \pm 4.48†	14.8 \pm 5.52†	3.9 \pm 2.36

* Decrease from preinjection concentration.

† Significant at 0.01.

‡ Significant at 0.025.

glucagon. The hyperglycemic response was greater and more prolonged in the diabetics than in the normals.²³ Analysis of lipoproteins showed no significant changes, possibly because of greater errors inherent in the method of estimation. A study of the effects of infusion of a larger dose of glucagon than those used in the present study or of repeated infusions might yield more definitive results.

The lipoprotein pattern of patients with diabetes for many years showed increased concentrations of L.D. β and β -lipoproteins. The abnormalities appear somewhat greater in stable than in brittle diabetics of middle age. The lipoprotein pattern of patients with intercapillary glomerulosclerosis is characterized by increased concentration of V.L.D. and L.D. β -lipoproteins, and usually a low concentration of α -lipoproteins. Infusion of glucagon into normal and diabetic subjects resulted in a transient but significant decrease in concentration of serum cholesterol, but no significant change in lipoprotein concentration.

Other Hormones

Other hormones, especially the sex hormones, play an important role in determining the lipoprotein pattern. The action of estrogens results in an in-

crease in α -lipoprotein concentration and the α : β lipoprotein ratio, while that of androgens causes an opposite effect. Numerous reports in the literature document these changes.²² Studies of the lipoproteins in some metabolic diseases involving endocrine imbalance, such as Addison's disease, Cushing's syndrome, acromegaly, are extremely limited, and the changes reported are variable.

HEPATIC FACTOR

Any change in hepatic metabolism, whether mediated by diet, disease processes, hormones, or other factors, may result in modification of the serum lipoprotein pattern since the liver is a site of synthesis of lipoproteins. The type and extent of hepatic damage will determine the concentration and composition of the lipoproteins. In terminal stages of hepatic failure, as observed in the hepatectomized dog,²⁴ the concentration of all lipoprotein fractions is decreased.

Studies in which the protein and methionine content of the diet was varied suggest that both may play an important role in regulatory elaboration of β -lipoproteins by the liver, since the concentration of β -lipoproteins was greatly decreased when diets low in these components were administered.²⁵ The concentration of L.D. β -lipoproteins was less affected than that of β -lipoprotein. These results provide an explanation for the low concentration of serum cholesterol and β -lipoprotein observed in some nutritional diseases. Olson²⁶ suggested that modification in serum lipid concentration produced by dietary means may occur because of changes that occur in the liver.

In infectious hepatitis,²⁷ biliary obstruction²⁸ and hepatic cirrhosis,²⁹ plasma lipids may be combined with peptides in an abnormal way to form compounds with physical and chemical characteristics that differ from those of normal lipoproteins. The ability of the liver to synthesize α -lipoproteins is impaired and the concentration of all fractions of density <1.063 may be modified, the type and extent or change depending upon the degree of hepatic involvement. In patients with infectious hepatitis the concentrations of serum cholesterol, β -, and low-density lipoproteins are frequently high, and an abnormal lipoprotein fraction with flotation rate —S 10–20 may be present (patient 1, TABLE 9). The abnormal serum lipoprotein pattern improves concomitant to improvement in hepatic function (patient 4, TABLE 9). This patient, who had post-necrotic hepatic cirrhosis when initially studied, had a serum cholesterol concentration of 94 and an α -lipoprotein concentration of 28 mg. per 100 ml. After 3 months of intensive therapy, the serum cholesterol had increased to 147 mg. per 100 ml. and the α -lipoprotein was normal. Three years later hepatic failure developed and the concentrations of serum cholesterol and β -lipoprotein were low and there was no measurable α lipoprotein. There is not always complete absence of α -lipoprotein in terminal hepatic failure (patient 5) but low concentrations of α - and β -lipoprotein are consistent findings.

EFFECT OF ERRORS IN PROTEIN AND COMPLEX PROTEIN METABOLISM ON SERUM LIPOPROTEINS

In agammaglobulinemia, a primary error in protein metabolism, the serum lipoprotein pattern was normal. A primary error of protein metabolism re-

TABLE 9
TYPICAL SERUM LIPOPROTEIN PATTERNS IN HEPATIC DISEASE

Diagnosis	Date of study	Clinical note	mg./100 ml. serum					
			-S70- 400	40-70	25-40	20-25	1-10	Chol- es- terol
Subacute infec- tious hepatitis	1956	Patient made good recovery	12	47	1250	305*	223	504
Chronic viral hep- atitis	1/1951	Results of hepatic function tests progressively worse	40	70	>500	5	117	300
	12/1953		141	164	>500	16	118	248
Hepatic cirrhosis (nutritional type)			28	82	180	5	122	113
Postnecrotic cir- rhosis	3-28-1955	Cirrhosis devel- oped after infec- tious hepatitis	0	84	246	0	28	94
	8-10-1955	Results of hepatic function tests slightly im- proved	24	30	218	7	204	147
	4-25-1958	Progressive he- patic failure	40	28	175	0	0	83
Laennec's cirrhosis (hepatic necro- sis)	6/1958 1956	Patient died Terminal studies	5	16	132	0	12	64

* -S10-20.

TABLE 10
SERUM LIPOPROTEIN PATTERNS OF PATIENTS WITH AMYLOIDOSIS

Patient, initials, sex	Date	Clinical notes	Lipoprotein concentration, mg./100 ml. serum					
			-S70-400	40-70	25-40	20-25	1-10	Chol- es- terol
McL. ♂		Localized amyloidosis of skin	40	30	164	9	184	199
I.K. ♂		Localized amyloidosis of skin	52	21	234	33	166	215
A.H. ♂		Amyloidosis of tongue	24	24	328	38	188	200
S.H. ♀	8/1954	Amyloid disease of stomach	47	38	230	14	171	160
	5/1955		14	14	250	19	94	198
	2/1956		19	40	282	7	136	204
	4/1956		19	38	247	9	126	208
	9/1954		24	66	1115	71	188	497
	10/1954		7	38	1000	19	75	320
	12/1954		16	152	940	35	143	360
R.Q. ♂		Died 12/1954. Generalized amyloidosis, evidenced wherever vessels or mus- cle were present Amyloid of liver, proved by biopsy. Bone marrow also showed amyloid	119	127	277	44	119	223

sulting in complete failure of synthesis of the protein moiety of α - or β -lipoprotein is theoretically possible. Perhaps the low concentration of β -lipoprotein observed in hereditary hyperglyceridemia represents a partial failure of synthesis of the β -protein moiety.

In patients in whom a metabolic error results in deposition of the complex protein, amyloid, serum lipoprotein patterns were essentially normal, unless the liver or kidneys were involved in the disease process (TABLE 10). Serial determinations made over a period of one year and a half on a patient (SH) with amyloid disease of the stomach,³⁰ showed normal or slightly low concentration of serum cholesterol and all lipoproteins. In contrast, patient HB, who had a nephrotic syndrome associated with amyloidosis, showed approximately 300 per cent increase in β -lipoprotein and slightly low α -lipoprotein concentrations.

SUMMARY

Among the factors that affect the serum lipoprotein pattern and its relation to metabolic disease, the important role of heredity, diet, hormonal balance and hepatic function have been elaborated.

Different heredity may result in some people having lipoprotein patterns significantly different from those of the "normal" white Americans. Thus the average $\alpha:(\beta + \text{low-density})$ lipoprotein ratios of normal Navajo and Peruvian Indians are higher than that of normal American white persons, while those of persons with hereditary hyperlipemia or hypercholesterolemia are lower. The incidence of atherosclerosis is greater in persons with hereditary hyperlipidemia than in normal white Americans, and is less in the normal American Indians, than it is in normal white Americans.

Administration of low-fat or vegetable-oil diets to patients with hyperlipemia was effective in lowering serum lipid concentrations of patients with lipoprotein patterns of the "hyperglyceridemia" or mixed hyperlipemia type and in maintaining them at nearly normal levels for as long as 4 years. In patients with greatly elevated concentrations of β and L.D. β -lipoproteins, the diets were less effective in maintaining lower serum lipid levels over long periods.

Hormonal balance is important to the maintenance of lipoproteins at normal levels. In patients with hypothyroidism, either increased concentration of all serum lipoproteins, or a primary increase in V.L.D. lipoprotein and a normal or low α -lipoprotein was observed. Treatment with thyroid hormone resulted in a return toward normal concentration of all lipoproteins. The lipoprotein pattern of middle-aged and old, stable, or brittle diabetics who had the disease 10 years or longer showed increased concentration of L.D. β and β -lipoproteins. Variations in lipoprotein concentrations within groups were great. Injection of glucagon into normal and diabetic subjects resulted in transient but significant decreases in concentrations of serum cholesterol, with insignificant changes in lipoprotein concentration.

The serum lipoprotein concentration was modified by hepatic damage. In hepatic failure the concentration of all lipoproteins was greatly decreased. In the primary defect of protein metabolism, agammaglobulinemia, serum lipoprotein concentrations were normal. In amyloidosis, the serum lipopro-

tein concentration was normal when the liver and kidneys were not involved in the amyloid process.

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